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Assessing the Hormone Response to High Intensity Exercise and Identifying Associations with Performance

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Assessing the Hormone Response to High Intensity Exercise and Identifying Associations with Performance

Rebecca Toone

A thesis submitted for the degree of Doctor of Philosophy

University of Bath

2015

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Abstract

The aim of this programme of research was to add to the existing body of knowledge on the measurement of certain hormones in relation to exercise training, the response to high intensity training, and their potential influence on short-term performance. The initial studies demonstrated agreement between venous and capillary concentrations of an array of hormones, and agreement between venous and saliva concentrations of testosterone and cortisol following scaling of saliva concentrations, suggesting suitability of use in an applied exercise setting. In addition, to ensure accurate measurement of steroid hormone concentrations in saliva, it was shown that samples should be refrigerated immediately, transferred to a freezer within 24 h of collection, and analysed within 28 days. Assessment of the response to two exercise bouts of a different type within the same day indicated that it could be beneficial to perform resistance training in the afternoon preceded by interval exercise in the morning in order to stimulate a hormonal milieu that may be more conducive to stimulating muscle protein turnover. The robust increases seen in testosterone and cortisol following interval exercise performed in the morning in that study were also observed in the same cycling sprint interval protocol performed in females. In this study, the magnitude of change in DHT concentration was related to sprint cadence. In investigating the potential acute effects of hormones on performance, the penultimate study demonstrated a positive association between affect as an indicator mood and percentage testosterone concentration during high intensity cycling. Conversely, in the final study, no postactivation potentiation effect was observed to different exercise stimuli, thus no association was observed between hormone concentrations and strength and power performance. These data may suggest that the acute short-term effects of hormone concentrations on performance may be more related to mood and behaviour in the context of this research.

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- Chapter 1 -

Introduction

Competitive athletes complete demanding training schedules, composed of different training modes that target different aspects of their performance. As performance standards within competitive sport continue to increase, the margins between success and failure are becoming increasingly small. Circulating hormone concentrations are known to change in response to exercise stimuli (Kraemer *et al*., 1990; Kraemer & Ratamess, 2005; Crewther *et al.,* 2006; Stokes *et al.,* 2013). Concentrations of certain of these hormones have been suggested to be important in adaptation to exercise training, as well as to providing an indication of the physiological state of athletes (Nindl *et al*., 2003; Kraemer & Ratamess, 2005; Crewther *et al.,* 2006; Beaven *et al.,* 2008a), and have also been shown to influence the performance of strength and power tasks, and associated with neuromuscular performance (Cardinale & Stone, 2006; Crewther *et al.,* 2009; Crewther *et al.,* 2012a; Crewther *et al.,* 2012b). In attempt to optimise training and performance, research has been conducted into the hormone response to exercise and whether these hormones can influence training adaptation and performance.

If hormones are to be routinely monitored alongside athletic training, establishing valid and reliable sampling methodologies that can be employed in an exercise setting is of utmost importance. Capillary blood and saliva collection methods are accepted as more convenient and less invasive methods that enable more frequent measurement of hormone concentrations outside of a laboratory setting. Despite frequent use, particularly of saliva sampling (Filaire *et al*., 2001; Elloumi *et al*., 2003; Beaven *et al*., 2008a; Beaven *et al*., 2008b; Crewther *et al*., 2008; Crewther *et al*., 2010; Edwards *et al*., 2006), few studies have sought to determine whether agreement between

concentrations of hormones measured in venous, capillary and saliva samples is maintained in response to a physiological stress. The applied training environment also provides challenges as to the handling and storage of samples following collection. Different storage conditions and durations prior to analysis are likely to influence hormone concentrations measured (Chikuma *et al.,* 1991; Kato *et al.,* 1992; Garde & Hansen, 2005; Wood, 2009). However, no explicit guidelines exist regarding the appropriate handling and storage of saliva samples following collection in order to preserve sample integrity. Establishing relationships between different sample media in an exercise setting, and the effect of different handling and storage procedures is an important step to ensure the measured concentrations are an accurate reflection of the changes in circulating hormone concentration.

In competitive sport it is common practice for athletes to complete at least two training sessions within a day. Depending on the event and requirements of the sport, these training sessions may target very different training goals. Different modes and intensities of training influence the subsequent changes in hormone concentration differently. As such, it is of importance to understand the hormone responses to individual exercise bouts of different types and the interaction between them. However, limited research has considered the influence of prior exercise on the hormone and performance responses to a subsequent bout. Additionally, while the hormone response to different resistance exercise schemes is well documented (Kraemer & Ratamess, 2005; Crewther *et al.,* 2006), few reports exist concerning the hormone responses to high intensity interval exercise despite this forming an important part of the majority of training programmes.

Recent research has provided compelling evidence that challenges the role of purportedly anabolic hormones in mediating gains in muscle mass and strength (West *et al.,* 2009; West *et al.,* 2010; West & Phillips, 2010). However, circulating concentrations of androgens also elicit short term effects and have been linked to enhanced performance of strength and power tasks (Cardinale & Stone, 2006; Crewther *et al.,* 2012a; Crewther *et al.,* 2012b) by exerting a rapid influence on neuromuscular function (Crewther *et al*., 2011a), an effect that has also previously been achieved via the use of conditioning stimuli generating post-activation potentiation (Hodgson *et al.,* 2005; Tillin & Bishop, 2009). Little research has sought to examine a link between conditioning stimuli and a possible associated hormone response that could be used to 'prime' or improve subsequent performance, an area with the potential to provide beneficial strategies within a competition environment.

Additionally, concentrations of testosterone and cortisol have been shown to be influenced by mood with elevated testosterone related to elements of athletic behaviour relation to confidence and motivation to compete (Kilduff *et al.,* 2013). Elevations in total and free testosterone concentration prior to performance have enhanced motivational behaviour, performance of identified skills (Cook & Crewther, 2012a), and strength performance (Cook & Crewther, 2012b). As a recent concept, further research is required to continue to investigate the possible link between mood and hormone concentrations and association with subsequent performance.

The studies comprising this thesis attempt to add to the body of literature concerning the measurement of hormones within the blood and in saliva in an exercise setting, as well as adding to the research concerning the response of hormones to different forms of

training, and the possible relationships between hormone concentrations and performance.

Specifically, it is intended that the investigations described in this thesis will fulfil the following objectives:

1. Establish, at rest and following exercise, the agreement between venous and capillary samples, and venous and saliva samples for concentrations of hormones frequently monitored in elite sport, and the suitability of their use.
2. Establish the effect of storage condition and duration on the concentration of selected steroid hormones typically analysed in elite sport, and best-practice recommendations for the preservation of sample integrity.

3. Assess the influence of bout order on the hormone responses to the performance of repeated sprint interval exercise and resistance exercise.

4. Assess the total testosterone and DHT response to a bout of repeated sprint cycle exercise in females, and the possible relationship with training status and performance.

5. Assess the hormone, performance, and psychological affect responses to a bout of repeated sprint cycle exercise when preceded by a high intensity period of cycling designed to induce fatigue; and establish whether any variation in mood, hormone response or performance between two trials was linked.

6. Establish whether different methods of priming or potentiating exercise would be successful in enhancing power and strength performance, and whether any potentiating effect was associated with elevations in hormone concentrations and muscular activation.

- Chapter 2 -

Review of Literature

2.1 Role of Hormones in Exercise

2.1.1 Long Term Effects

2.1.1.1 Adaptation

Elevations in concentrations of hormones such as testosterone, growth hormone (GH) and insulin like growth factor (IGF-1) following exercise have been reported to contribute to the gain in muscle hypertrophy and strength that are observed with resistance training (McCall *et al.,* 1999; Kraemer & Ratamess, 2005; Ratamess *et al.,* 2009; Spiering *et al.,* 2009). As the primary anabolic hormone, testosterone in particular has been associated with gains in muscle strength and mass based on observations that muscle growth occurs at puberty when testosterone concentrations increase (Ramos *et al.,* 1998; Mauras, 2001), and supported by research demonstrating reversal of the gradual loss of muscle strength and mass that occurs with ages following exogenous administration of testosterone (Anawalt & Merriam, 2001), and attenuated gains in strength with resistance training after suppression of endogenous testosterone production (Kvorning *et al.,* 2006). Additionally, strength gains have recently been reported when athletes trained according to resistance exercise protocols that stimulated their individual greatest increase in testosterone concentration (Beaven *et al.,* 2008b).

There are consistent reports throughout the literature relating to the ability of the anabolic hormones testosterone, IGF-1, and growth hormone to contribute to gains in muscle mass and strength when stimulated through exercise (McCall *et al.,* 1999; Kraemer & Ratamess, 2005; Crewther et al., 2006; Ratamess *et al.,* 2009; Spiering *et*

al., 2009). Recently, clear associations have been demonstrated between the magnitude of testosterone response elicited by training and subsequent gains in muscle mass an strength (Beaven *et al.,* 2008a; 2008b). In the first of two studies, elite rugby players demonstrated individual responses to four different resistance exercise protocols. In a subsequent investigation utilising a crossover design, two groups of players spent two three week blocks training according to the protocol that elicited either their individual maximum or minimum testosterone response. When training according to the protocol that elicited their individual maximum testosterone response for a period of three weeks, all athletes exhibited increases in bench press and leg press 1 RM as well as body mass. While training according to the protocol eliciting individual minimum testosterone response 75% of athletes demonstrated either no increase, or a reduction in 1 RM performances. Using similar protocols, Hansen *et al.* (2001) and Ronnestad *et al.* (2011) endeavoured to manipulate the internal hormonal milieu in attempt to further elucidate the effect of transiently elevated endogenous hormones on strength training adaptations. In both studies, additional lower body exercise was performed in order to increase endogenous hormone concentrations prior to training of the elbow flexors. Hansen *et al.* (2001) utilised a two group design with one group performing the additional lower body exercise, while the opposing group did not. In the study of Ronnestad *et al.* (2011) all participants completed four training sessions of the elbow flexors each week, two of which were preceded by additional leg exercise and two of which were not, with opposing arms being trained under each condition. Following a training period, increases in 1RM, peak power and muscle volume were observed only in the arm that was trained following leg exercise, under 'high hormone' conditions (Ronnestad et al., 2011), while observed increases in arm strength were 9% in participants solely training

the upper body, and 37% in those who completed the additional preceding leg exercise (Hansen *et al.,* 2001).

While the studies discussed above identify an association between transient elevations of hormones and the contribution to gains in muscle mass and strength, more clinical work has also identified association between elevations in hormone concentrations and activation of the signalling pathways responsible for increases in muscle hypertrophy. Figure 2.1 illustrates some of the pathways these hormones are reported to have an effect. Following resistance exercise, or other forms of muscular overload, enhanced translation mediates the initial increase in protein synthesis. (Chesley *et al.,* 1992; Welle *et al.,* 1999). The primary pathway involved in this process involves Protein kinase B (Akt). Akt signals the response to muscular overload by phosphorylating downstream translation initiation factors to increase translational efficiency and protein synthesis (Bodine *et al.,* 2001; Kimball *et al.,* 2002). As demonstrated in Figure 2.1, phosphorylation of Akt, allows subsequent phosphorylation of key downstream target mammalian target of rapamycin (mTOR), which activates stimulators of protein synthesis: 70 k-Da ribosomal protein S6 kinase (p70S6k), and eukaryotic initiation factor 2-beta (eIF2B); and by repressing inibitors: glycogen synthase 3-beta (GSK-3b) and 4E binding protein 1 (4E-BP1) (Reynolds *et al.,* 2002). While muscle contraction can directly activate Akt, hormones exert their effect on these pathways by binding to cell surface receptors and exerting signalling effects via activation of phosphatidylinositol-3 kinase (PI-3K) (Spiering *et al.,* 2008). While increases in IGF-1 and testosterone stimulate Akt signalling, cortisol opposes it. Attenutation of p70S6k phosphorylation that was likely due to cortisol inhibition has been observed following resistance exercise stimulating large increases in cortisol (Spiering *et al.,* 2008). As

Figure 2.1 also demonstrates, testosterone is also able to exert influence on muscle protein synthesis in a more direct manner via androgen receptors (AR). Testosterone binds to and converts AR to a transcription factor capable of translocating to the nucleus and associating with DNA to regulate gene expression (Spiering *et al.,* 2008).

Following administration of testosterone to cultured cells, increases in phosphorylation of signalling proteins have frequently been reported. Akt phosphorylation has been shown to be increased 15 min after testosterone administration to rat skeletal muscle myotubes, with a subsequent increase in p70S6K at 60 min (Basualto-Alarcon *et al.,* 2013). Morphological changes were also noted during this study, with an increase in myotube cross sectional area observed. However, both p70S6k phosphorylation and hypertrophy were abolished by Akt and mTOR inhibition, with hypertrophy additionally prevented by AR inhibition. Similar findings were detected following administration of both testosterone and DHT to rat myoblasts (Wu *et al.,* 2010). Testosterone administration elevated mTOR phosphorylation within 20min and p70S6k phosphorylation was elevated within 2 h. Hypertrophy was again noted in the cells treated with both testosterone and DHT, and was not observed in cells lacking AR, or followoing administration of and AR antagonist. Similar findings to these are present throughout the literature (Nguyen *et al.,* 2005; Sato *et al.,* 2008; Altamirano *et al.,* 2009; Fu *et al.,* 2012; White *et al.,* 2013). Associations between testosterone and muscle signalling have also been reported in human subjects completing resistance exercise (Spiering *et al.,* 2009). Similar to protocol previously mentioned, high volume upper body resistance exercise was used to elicit elevated concentrations of testosterone prior to performance of leg exercise during one of two trial, and was not performed in other which acted as the control. Testosterone concentrations were 16% greater during the

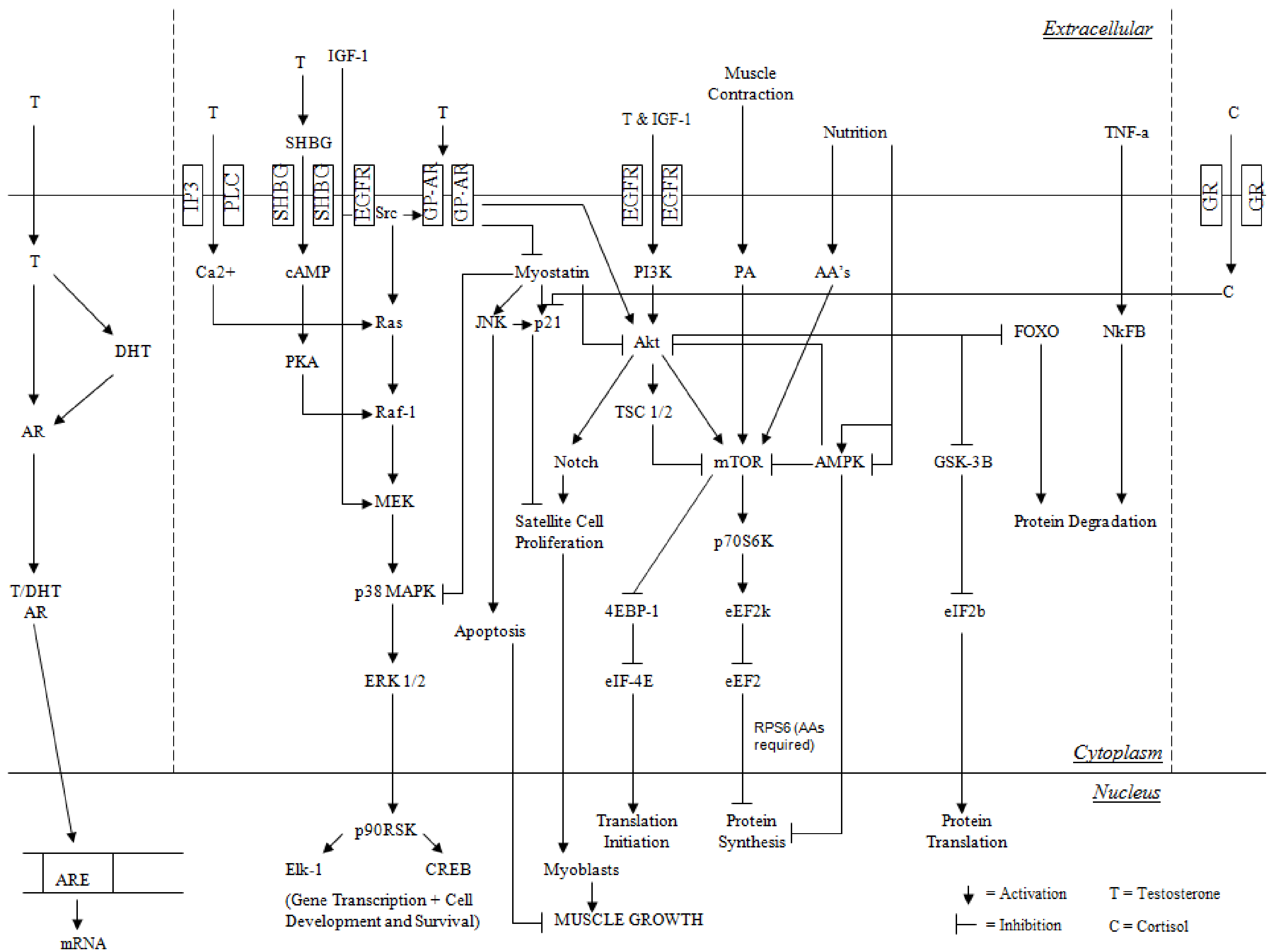

Figure 2.1 Diagram demonstrating the major signalling pathways through which testosterone, IGF-1 and cortisol are reported to exert an effect

'high hormone' trial, and this was associated with greater muscle AR receptor content at 3 h post exercise (Spiering *et al.,* 2009).

The administration of exogenous testosterone and suppression of endogenous testosterone in humans has provided further evidence of the association between circulating testosterone concentrations and gain in muscle mass and strength. Among hypogonadal men 10 weeks of treatment with testosterone replacement, bringing endogenous concentrations back to within normal physiological range, and alongside controlled energy intake and protein composition, resulted in an increase in fat-free mass and muscle size and strength (Bhasin *et al.,* 1997). Similar observations were also observed following three years of testosterone replacement, with increases in fat free mass of 3.1 kg, principally occurring within the first 6 months of treatment (Snyder *et al.,* 2000). Dose response relationships have also been observed in healthy eugonadal between the degree of testosterone supplementation and subsequent gains in muscle mass and strength. Changes in fat-free mass increased in a dose dependent manner from 3.4 - 7.9 kg, with changes in leg power and thigh and quadriceps volume strongly correlated with measured testosterone concentrations (Bhasin *et al.,* 2001). While exogenous testosterone alone increases muscle size and strength, the effect is increased when administration of testosterone is combined with resistance exercise (Bhasin *et al.,* 1996). Increases in fat free mass with the administration of testosterone combined with resistance exercise were 6.1 kg, nearly double that of administration of testosterone alone (3.2 kg) (Bhasin *et al.,* 1996). Increasing doses of testosterone have also been demonstrated to increase the number of myonuclei per muscle fibre, leading to the assertion that the testosterone induced increases in muscle volume are true changes due to muscle fibre hypertrophy (Sinha-Hikim *et al.,* 2002). Furthermore, gains in strength

following resistance training are attenuated when endogenous testosterone is suppressed (Kvorning *et al.*, 2006). In a double blinded study, two groups of trained males completed eight weeks of resistance training, with one group administered with a GnRH analog and the other a placebo. Testosterone decreased significantly in the males receiving the testosterone suppressant, who also demonstrated no change in knee extension strength following the training period. Testosterone concentration did not change in the placebo group and significant increase in strength were observed. In addition body fat mass actually increased in the suppressant group, while a decrease was observed in the placebo group (Kvorning *et al.,* 2006).

More recent research has, however, challenged these views, questioning the importance of purportedly anabolic hormones in muscular adaptation and gains in muscle mass and strength. Myofibrillar protein synthesis, required for muscle hypertrophy, was not enhanced by simultaneous elevations in circulating concentrations of testosterone, GH and IGF-1 (West *et al.,* 2009). Male participants completed unilateral resistance exercise of the elbow flexors of the elbow flexors on two separate occasions. On one occasion, the exercise was followed by high volume leg resistance exercise in order to stimulate an increase in endogenous hormone concentrations in order to compare muscle protein synthesis in the hours following resistance exercise between basal, or low, hormone concentrations and high hormone concentrations. In both trials the exercise was followed by consumption of 25g of protein. Marked elevations were observed in testosterone, IGF-1 and GH in response to the high hormone trial but not in the low hormone trial. The exercise protocol stimulated an increase in muscle protein synthesis in both trials as well as phosphorylation of p70S6k with no effect of hormone concentrations (West *et al.,* 2009). In a study design similar to that of Ronnestad *et al.*

(2011) the same authors subsequently investigated the influence of elevated hormone concentrations on changes in muscle size and strength across a training period (West *et al.,* 2010). Utilising the same protocol as the earlier study (West *et al.,* 2009), and conversely to the study of Ronnestad *et al.* (2011), the high volume leg exercise was performed after the elbow flexor exercise. Participants trained for 15 weeks with 72h between each session, and alternating between high and low hormone conditions. Increases in testosterone, IGF-1 and GH were detected at 15 min and 30 min post exercise in the high hormone sessions only, and was maintained across the training period. Following the training period, and in opposition to the findings of Ronnestad *et al.* (2011), muscle cross sectional area was increased to the same extent in both arms. The authors directly challenged the findings of Ronnestad *et al.* (2011) by subsequently demonstrating that performance of high volume leg exercise before or after the arm resistance did not influence the observed responses either (West *et al.,* 2013). Hormone delivery to the biceps was estimated via brachial artery blood flow. Hormone concentrations peaked at 15 min post leg exercise in both trial orders, but no difference in magnitude or blood flow was found between trial orders.

Adaptation to resistance exercise has instead been attributed to the stimulus of loaded muscle contraction and amino acid availability, rather than the transient increase in endogenous hormone concentrations (West & Phillips, 2010). Wilkinson *et al.* (2006) demonstrated occurrence of local muscle hypertrophy in the quadriceps in the absence of changes in circulating hormone concentrations. Participants trained unilaterally three times per week for eight weeks, completing three sets of six to ten repetitions of knee extension and leg press at 80 - 90% of 1 RM. 1 RM and muscle cross sectional area increased in the trained limb over the course of the training period despite no increase

being observed in testosterone in response to training (Wilkinson *et al.,* 2006). Additionally, the feeding of protein or amino acids has been shown to stimulate muscle protein synthesis, an effect that seems to be due to the amino acids themselves (Greenhaff *et al.,* 2008). Intravenous infusion of amino acids has been demonstrated to increase post-exercise muscle protein synthesis rates and prevent the exercise-induced increase in protein degradation (Biolo *et al.,* 1997). Even in the absence of prior resistance exercise, similar intravenous infusion of amino acids , muscle protein synthesis rates rapidly increase (Volpi et al., 1998). In a more applied and practical manner, the ingestion of protein or amino acids following exercise has also elicited increases in muscle protein synthesis, however, the effect appears to be dose dependent. Ingestions of a large amount (30 - 40g) of amino acids following exercise has been shown to stimulate muscle protein synthesis (Tipton *et al.,* 1999), while smaller doses of essential amino acids (6 g) stimulated a small increase for a period of 2 h prior to protein balance becoming negative (Borsheim *et al.,* 2002). Amino acids also exert their effect via the signalling pathways demonstrated in Figure 2.1. Increase amino acid concentrations, particularly leucine, activate mTOR and p70S6K, increasing the rate of mRNA translation (Rasmussen *et al.,* 2000). Indeed, it seems that ingestion of amino acids following resistance exercise stimulates an additive increase in muscle protein signalling induced by the resistance exercise stimulus (Koopman et al., 2007). In combination with their observations demonstrating no increase in muscle protein synthesis, or in muscle size or strength despite increases in endogenous hormone (West *et al.*, 2009; West & Phillips, 2010), West et al. argue that these factors are primarily responsible for governing muscular adaptation. With conflicted findings and opinions associated with the role of exogenous hormones, assertions have been made for a

stronger role for the short term effects of these hormones, particularly on mood and/or behaviour and performance.

2.1.2 Short Term Effects

2.1.2.1 Signalling

The long term effects of androgens are thought to be regulated by steroid receptors found in the cytosol of target cells that control transcription of DNA (Heinlein & Chang, 2002). Further steroid receptors have, however, been located on the cell membrane comprised of G-proteins, protein kinases, neuro-transmitters and ion channels (Falkenstein *et al.,* 2000). Although the precise role of these receptors still remains to be clarified it does not appear to be related to gene transcription, with the non-genomic actions elicited occurring on a much shorter timescale (Makara & Haller, 2001; Heinlein & Chang, 2002). Within seconds, the effects of steroid binding at the cell membrane receptors induces second messenger signalling the intracellular release of ions including ATP, calcium and potassium (Passaquin *et al.,* 1998; Jaimovich & Espinosa, 2004; Han *et al.,* 2005). Subsequent downstream effects have been observed on protein kinase pathways (Estrada *et al.,* 2003; Han *et al.,* 2005; Nguyen *et al.,* 2005). These signalling effects can influence neuronal inhibition or excitation within minutes (Smith *et al.,* 2002; Zaki & Barrett-Jolley, 2002). Hormonal stimulation of these signalling process could therefore influence the neuromuscular system and associated tissues.

Protein kinase pathway activation resulting from steroid hormone binding at the cell membrane receptors has been demonstrated to influence force production within muscle fibres. Following DHT administration, enhancement of force production in fast twitch

skeletal muscle fibres has been observed, and was accompanied by increases in MAPK and ERK 1/2 phosphorylation (Hamdi & Mutungi, 2010). The effect was not apparent in slow twitch muscle fibres.

Release of calcium into skeletal muscle cells triggered by steroid hormone binding at the cell membrane receptors identifies a further role for steroid hormones linked to acute muscular function and performance. Release of calcium not only instigates muscle contraction but it is also involved in twitch relaxation, energy metabolism and the structural integrity of the muscle fibre in muscle fibre (Berchtold *et al*., 2000). In addition, steroid hormones and growth factors may assist in the mediation of muscle contraction via modification of activity of the sodium:potassium pump. IGF-1 has been shown to stimulate an influx of potassium and efflux of sodium within the muscle cells, while glucocorticoid administration *in vitro* appears to up-regulate sodium potassium pump content in muscle cells (Clausen, 2003).

The above evidence indicates a role for steroid hormones, in particular testosterone and DHT in influencing acute muscle contraction and force production, and suggests the potential short term effects of steroid hormones on performance are more concerned with expression of muscular force and power.

2.1.2.2 Neuromotor System

Muscle function can also be mediated via steroid hormones effects on the motor system. Low free cortisol concentrations have been associated with enhanced motor cortex response, while administration of hydrocortisone prevented a response (Sale *et al.,* 2008). In addition, exogenous testosterone administration has been reported to decrease

the cortical motor threshold in humans (Bonifazi *et al.,* 2004). These effects have been linked to improved performance via the influence on skeletal muscle function. Manipulation of testosterone concentration via administration of exogenous testosterone has been shown to influence strength and leg power (Rogerson *et al.,* 2007; Storer *et al.,* 2003), with administration of cortisol resulting in a decrease in leg power (Fitts *et al.,* 2007).

2.1.2.3 Mood, Behaviour & Cognition

Modifications in behaviour have been observed within minutes following manipulation of testosterone and cortisol concentrations in animal studies, indicating a non-genomic, short-term role for steroid hormones in the mediation of mood and behaviour. Administration of testosterone and DHT in animal studies has been shown to rapidly influence cognition and behaviour (Aikey *et al*., 2002, James & Nyby, 2002; Lacreuse *et al.,* 2009). In humans, the effects of the use of androgenic-anabolic steroids on mood and behaviour have been well documented with reported increases in aggression and/or hostility, and changes in mood (Hartgens & Kuipers, 2004). High levels of negative affect have also been reported in hypogonadal patients (O'Connor *et al.,* 2002).

Steroid hormones have also been shown to influence cognitive function in humans with demonstration of administration of testosterone improving verbal fluency in males (O'Connor *et al.,* 2001) and improving visuospatial ability in females (Aleman *et al.,* 2004). Administration of cortisol on the other hand has been shown to compromise memory (Buss *et al.,* 2003), increase risky decision making (Putman *et al.,* 2010), and increase error rates during tasks such as the stroop test (Hsu *et al,* 2003). These effects have been attributed to the influence of steroid hormones on the brain, and alterations in

brain neuronal activity. However, the precise location of action of steroid hormones within the brain is unknown. All these effects have the potential to influence physical performance through influencing motivation and behaviour, as well as influencing decision making and behaviour than can affect execution of skills.

2.1.2.4 Substrate Metabolism

As a glucocorticoid, one of the primary roles of cortisol is to mediate gluconeogenesis and glycogenolysis through the metabolism of lipid, protein and glycogen, as well as through permissive action on other hormones such as catecholamines (Gorostiaga et al., 1988; McNurlan et al., 1996; Rooyackers & Nair, 1997; Viru & Viru, 2004). Testosterone has been shown to promote rapid increase in metabolism and uptake of glucose in myotubules *in vitro* (Tsai & Sapolsky, 1998) and also to regulate insulin sensitivity in rats (Holmang & Bjorntorp, 1992), yet no difference were observed in substrate use during endurance performance in individuals with high, normal or low testosterone concentrations (Braun *et al.,* 2005)

Substrate metabolism at rest and during exercise may also be mediated by estrogen. At rest, elevated concentrations of estrogen have been associated with greater muscle glycogen content and, therefore, glycogen sparing (Hackney, 1990), and also with elevated levels of plasma free fatty acids and a lower respiratory exchange ratio (Reinke *et al.,* 1972; Nicklas *et al.,* 1989). During exercise, elevated concentrations of estrogen may enhance fatty acid oxidation and utilisation and spare hepatic glycogen, and have been associated with enhanced run time to exhaustion in rats (Kendrick *et al.,* 1987; Hatta *et al.,* 1988). In contrast, the dual administration of estrogen and progesterone elicited greater glucose utilisation.

2.2 Acute Hormone Response to Exercise

2.2.1 Resistance Exercise

Resistance exercise is the mode of exercise that has been most frequently researched in terms of the hormone response to exercise. The hormone response to resistance exercise is influenced by the exercise protocol used. Resistance exercise protocols most frequently utilised in resistance training with the aim of mediating muscle strength, size and power form three broad schemes. These are: hypertrophy, characterised by moderate loads and repetitions, controlled movement and short rest periods; maximal strength/neuronal, characterised by heavy loads, low repetitions, maximal efforts, and long rest periods; and dynamic power, characterised by light loads, explosive/ballistic movement, and moderate rest periods. In addition to the influence of programme design on hormone response to resistance exercise, other factors may also influence the observed response. These factors include programme design, age, training status, and nutrition, and will also be considered in the following sections.

2.2.1.1 Testosterone

Hypertrophy resistance exercise schemes tend to elicit large increases in testosterone concentrations, above those observed in response to maximal strength and dynamic power protocols (Crewther et al., 2008). In a direct comparison between hypertrophy and maximal strength protocols, the increase in circulating testosterone in response to the hypertrophy protocol was nearly 3-fold greater (Kraemer *et al.,* 1991), while in a different study, an increase in testosterone following completion of a hypertrophy protocol, with no change observed following completion of a maximal strength protocol (Hakkinen & Pakarinen, 1993). However, the magnitude of increase in testosterone to hypertrophy protocols is variable. Although, a lone observation, no change in

Table 2.1 Summary of the acute hormonal responses to hypertrophy protocol resistance exercise in males.

Author	Subjects (n)	Training Status	Protocol	Change in Hormone Concentration		
				T	IGF-1	C
Kraemer *et al.*, (1991)	8	Trained	8 exercises: 3 x 10 (10 RM)	+ 72%	+ 34%	
Kraemer et al., (1993)	8	Trained	8 exercises: 3 x 10 (10 RM)			+ 65%
Hakkinen & Pakarinen (1993)	10	Trained	1 exercise: 10 x 10 (10 RM)	+ 24%		+ 149%
Chandler *et al.,* (1994)	9	Trained	8 exercises: 2 x 8-10 (75% 1 RM)	+ 20%	Nil	
McMurray *et al.*, (1995)	8	Trained	6 exercises: 3 x 6-8 (80% 1RM)	+ 21%		Nil
Gotshalk *et al.,* (1997)	8	Trained	8 exercises: 1 x 10 (10 RM)	+ 14%		+ 10%
			8 exercises: 3 x 10 (10 RM)	+ 32%		+ 25%
Volek *et al.,* (1997)	12	Trained	1 exercise: 5 x 10 (10 RM)	+ 7%		Nil
Bosco *et al.,* (2000)	6	Trained	3 exercises: 12 x 8-12 (70-75% 1 RM)	- 70%		
Smilios *et al.,* (2003)	11	Trained	4 exercises: 2 x 10 (75% 1 RM)	Nil		Nil
			4 exercises: 4 x 10 (75% 1 RM)	+ 10%		+ 28%
			4 exercises: 6 x 10 (75% 1 RM)	Nil		+ 27%
Zafeiridis *et al.,* (2003)	10	Trained	4 exercises: 4 x 10 (75% 1 RM)			+ 38%
Rubin *et al.,* (2005)	9	Trained	1 exercise : 6 x 10 (10 RM)		+ 11%	
McCall *et al.,* (1999)	8	Recreational	8 exercises: 3 x 10 (10 RM)	Nil	Nil	+ 27%

Table 2.1 continued

Author	Subjects (n)	Training Status	Protocol	Change in Hormone Concentration		
				T	IGF-1	C
Kraemer *et al.,* (2001)	10	Recreational	10 exercises: 3 x 10 (10 RM)	- 29% *		
McGuigan *et al.,* (2004)	8	Recreational	2 exercises: 6 x 10 (75% 1 RM)			+ 97% *
Vanhelder *et al.,* (1985)	5	Un-Trained	1 exercise: 7 x 10 (10 RM)			+ 80%
Kraemer *et al.,* (1992)	8	Un-Trained	4 exercises: 3 x 10 (10 RM)	Nil	Nil	
Hakkinen & Pakarinen (1995)	8	Un-Trained	1 exercise: 5 x 10 (10 RM)	+ 9%		Nil
Kraemer *et al.,* (1998)	8	Un-Trained	1 exercise: 4 x 10 (10 RM)	+ 38%		+ 78%
Hakkinen *et al.,* (1998)	10	Un-Trained	2 exercises: 4 x 10 (100% MVC)	+ 27%		Nil
Kraemer *et al.,* (1999)	8	Un-Trained	1 exercise: 4 x 10 (10 RM)	+ 37%		+ 80%
Hakinnen *et al.,* (2000)	10	Un-Trained	1 exercise: 5 x 10 (10 RM)	+ 21%		
Rubin *et al.,* (2005)	10	Un-Trained	1 exercise : 6 x 10 (10 RM)		+ 10%	

(T = Testosterone, C = Cortisol, * Salivary Hormones)

testosterone in response to this type of protocol has been reported (Smilios *et al.,* 2003), while increases of 72% have also been reported (Kraemer *et al.,* 1991). The testosterone response to dynamic power resistance exercise protocols appears to be the most consistent (Mero *et al.,* 1991; Pullinen *et al.,* 1998; Volek *et al.,* 1997).

The variability seen in response between the different forms of resistance exercise training are likely to be linked to the variation in the protocols used within each form. As shown in Tables 2.1 - 2.3 there is much greater variation in the protocols used when assessing the response to hypertrophy or maximal strength resistance training than to power resistance training. Hypertrophy protocols have varied from completing four sets of ten repetitions of a single exercise (Kraemer *et al.,* 1999) to three sets of ten repetitions of eight exercises (Kraemer *et al.,* 1991; Kraemer *et al.,* 1993; Gotshalk *et al.,* 1997). Maximal strength protocols vary from completing twenty single repetitions of a single exercise (Hakkinen & Pakarinen, 1993), to completing five sets of five repetitions of eight exercises (Kraemer *et al.,* 1990), while protocols used to assess the hormonal response to power protocols vary much less, ranging from ten sets of six repetitions of a single exercise (Mero *et al.,* 1991; Mero *et al.,* 1993; Pullinen *et al.,* 1998), to five sets of ten repetitions of a single exercise (Volek *et al*., 1997). This consistency in protocol is accompanied by a consistent testosterone response from 13 - 18%, while the observed response from maximal strength protocols has ranged from 0 - 30%. The testosterone response to a given session appears to be linked to protocol volume. The greater volume of work completed during the study of Kraemer *et al.* (1990) stimulated a 30% increase in total testosterone, the study of Hakkinen & Pakarinen (2000) did not detect an increase in testosterone, while a study with a total volume between these two protocols, consisting of three sets of three to six repetitions

of three exercises, generated an increase in testosterone of 17% (Raastad *et al.,* 2000). This would suggest repetition volume may be a key component to generating a testosterone response.

Testosterone response to all of the protocol appears to be related to age and training status of the participants. As demonstrated in Table 2.3, greater testosterone responses to maximal strength type resistance exercise has been observed in males in their mid-twenties in comparison to those in their teens (Mero *et al.,* 1993; Pullinen *et al.,* 1998). However this difference was offset by reducing the rest period between sets, providing support for the assertions and observations that there is a strong metabolic component to stimulation of testosterone production and release (Lu *et al.,* 1997). A training history providing weightlifting experience of more than two years in youth participants has also been shown result in a greater testosterone response that in those with less experience (Craig *et al.,*1989). Training status also appears to influence the response within adult males. Following 12 weeks of resistance training a 12% increase in testosterone was observed in previously untrained males that had not demonstrated a testosterone response to the same resistance exercise session performed before the training period (Kraemer *et al.,* 1998). Strength trained males have also been shown to respond to a greater extent to a given resistance exercise session than untrained individuals (Ahtianen *et al.*, 2004; Tremblay *et al.,* 2004), however, the inverse has also been reported (Ahtianen *et al.,* 2003). Interestingly, in individuals training specifically to increase muscle mass an inhibited hormone response to resistance exercise has been observed (Bosco *et al.,* 2000). This may help explain some of the variation in response seen, particularly within hypertrophy resistance training.

Nutritional strategies may also influence the hormone response to resistance exercise. Carbohydrate and/or protein supplementation immediately after and 2 h after exercise resulted in a decrease in total testosterone concentration in the post-exercise period (Chandler *et al.,* 1994). Similar observation have also been reported following carbohydrate and protein supplementation across three days (Kraemer *et al.,* 1998) and in response to a mixed meal, a supplement of similar nutritional content, and carbohydrate only (Bloomer *et al.,* 2000). This response has been suggested to be due to an increase in hormone uptake or greater clearance from the circulation as it has not been accompanied by a decrease in LH (Chandler *et al.,* 1994). It appears that nutritional considerations are important in regulating the testosterone response to exercise.

2.2.1.2 Cortisol

Similar to observations in testosterone, hypertrophy resistance exercise protocols have been shown to generate greater increases in cortisol concentration than maximal strength or dynamic power protocols. Increases in cortisol concentration as great as 175% have been observed following hypertrophy protocols (Mulligan *et al.*, 1996), while no response of cortisol has frequently been reported following performance of maximal strength protocols (Kraemer *et al.,* 1993; Raastad *et al.,* 2000; Smilios *et al.,* 2003; Zafeiridis *et al.,* 2003). Dynamic power resistance exercise protocols have also resulted in increase in cortisol concentration (Mero *et al.,* 1991), but not of the same magnitude as hypertrophy protocols. Indeed, on average the increase in cortisol concentration to hypertrophy resistance exercise is 45%, and 20% in response to dynamic power protocols (Crewther *et al.,* 2006).

As with testosterone, these differences may be due to the protocol and design of the resistance exercise session appears to be important in determining the cortisol response. Session volume and load appear to be an important determining factor, with hypertrophy protocols typically containing a higher volume of work of up to three sets of ten repetitions of eight exercises (Kraemer *et al.,* 1991; Kraemer *et al.,* 1993; Gotshalk *et al.,* 1997) than dynamic power protocols which generally consist of ten sets of six repetitions of a single exercise (Mero *et al.,* 1991; Mero *et al.,* 1993; Pullinen *et al.,* 1998), and in turn contain a higher volume of work than maximal strength protocols such as twenty single repetitions of a single exercise (Hakkinen & Pakarinen, 1993), or five sets of five repetitions of eight exercises (Kraemer *et al.,* 1990). This also suggests that cortisol response is linked to the metabolic demand and intensity of the resistance exercise session. Indeed, significant correlations have been observed between circulating lactate and cortisol concentrations (Kraemer *et al.,* 1989; Ratamess *et al.,* 2005). Increasing the intensity and demand of a session by reducing the rest period between sets also result in higher elevations in cortisol concentration (Kraemer *et al.,* 1987; Kraemer *et al.,* 1993; Kraemer *et al.,* 1996).

The relationship between the cortisol response to resistance exercise and the training status of an individual is requires further elucidation, with the literature demonstrating conflicting findings. Greater cortisol responses have been reported in untrained males following a lower body resistance exercise session (McMillan *et al.,* 1993), with a reduced cortisol response to a given session following 12 weeks of resistance training in previously untrained males (Kraemer *et al.,* 1999). However, other research has found no difference in cortisol response to resistance exercise in untrained and strength trained men (Ahtiainen *et al.,* 2004, Tremblay *et al*., 2004), no correlation between response

and length of resistance training experience (Kraemer *et al.,* 1992), and also no change in cortisol response following a training period (Kraemer *et al*., 1998; Kraemer *et al.,* 1999). However, similar to testosterone, cortisol response does appear to be influence by the type of training experience. In endurance trained males, cortisol response to a given resistance exercise session at a given relative percentage is attenuated in comparison to strength trained counterparts (Tremblay *et al* 2004). Differences in muscle size, strength and composition, and energy metabolism may explain these findings.

Nutritional practices appear less important in regulating the cortisol response to resistance exercise. No difference in cortisol has been observed following either carbohydrate supplementation or a placebo prior to, and immediately after, a resistance exercise session (Thyfault *et al*., 2004)). Several other investigations have also failed to detect in cortisol response performed with carbohydrate or protein supplementation versus a placebo (Fry *et al.,* 1993; Bloomer *et al.,* 2000; Williams *et al.,* 2002).

2.2.1.3 GH & IGF-1

Changes in IGF-1 concentration following resistance exercise have been observed, but unlike testosterone and cortisol, the influence of the resistance exercise protocol used seems to influence the response less with consistency in the degree of increase in IGF-1 concentration observed following both hypertrophy and maximal strength resistance exercise protocols (Kraemer *et al,* 1990; Kraemer *et al.,* 1991; Raastad *et al.,* 2000; Rubin *et al.,* 2005). It has been suggested that influence of resistance exercise on IGF-1 may in part be governed by the partitioning of IGF-1 among its binding proteins with resistance exercise also eliciting changes in binding protein concentrations (Nindl *et al.,*

2001). In contrast, a marked difference between resistance exercise protocol is apparent in the GH response to resistance exercise with hypertrophy protocol generating vast increase in circulating GH concentrations up to 14-fold (Kraemer *et al.,* 1990; Kraemer *et al.,* 1991; Hakkinen & Pakarinen, 1993, Raastad *et al.,* 2000; Zafeiridis *et al.,* 2003), with significantly smaller increases of between 3 and 4-fold in response to maximal strength protocols measured in the same subjects (Kraemer *et al.,* 1990; Zafeiridis *et al.,* 2003).

The influence of resistance exercise training type or protocol on the IGF-1 response to a bout of resistance exercise is less clear than for testosterone or cortisol. As Tables 2.1 & 2.2 demonstrate, the responses to hypertrophy and maximal strength type resistance exercise are varied. In response to a maximal strength protocol a decrease of 7% has been observed (Raastad *et al.,* 2000), as have increases of 25 % (Kraemer *et al.,* 1990) and 27 % (Kraemer *et al.,* 1991). In response to hypertrophy resistance exercise, no change (Kraemer *et al.,* 1992), and increases of 10% (Rubin *et al.*, 2005) have been observed. With the limited number of studies to have considered IGF-1 it is difficult to draw comparison. It is suggested differences in response are likely due to other factors such as a possible increase in IGF-1 uptake, different release mechanisms of the growth factor from liver and from muscle (Crewther *et al.*, 2006), and also the way it is partitioned among its binding proteins (Nindl *et al.,* 2001) .

Given the discussion above it is therefore unsurprising that training status appears to have little influence on the IGF-1 response to resistance exercise. Indeed, in response to hypertrophy resistance exercise trained and untrained males demonstrated increase in IGF-1 concentration of 11% and 10% respectively when lifting the same relative load

(Rubin *et al.,* 2005). However, the absolute concentrations were consistently higher in the resistance trained males. This may suggest difference in basal concentrations of IGF-1 with resistance training. Greater basal level of IGF-1 have indeed been reported in active individuals above those who were sedentary (Kostka *et al.,* 2003).

Few studies have examined the influence of nutritional supplementation on IGF-1 concentration alone as well as in relation to exercise. Elevated IGF-1 concentrations have however been observed with carbohydrate and protein supplementation alongside three consecutive days of resistance exercise (Kraemer *et al.,* 1998). It is possible feeding may influence the IGF binding protein concentrations, and the expression of muscle IGF-1. Indeed, it is thought that locally produced IGF1 in the muscle may exert metabolic effects and possible stimulate glucose uptake (Berg & Bang, 2004).

Table 2.2 Summary of the acute hormonal responses to maximal strength protocol resistance exercise in males.

Author	Subjects (n)	Training Status	Protocol	Change in Hormone Concentration		
				T	IGF-1	C
Kraemer *et al.*, (1990)	9	Trained	8 exercises: 3/5 x 5 (5 RM)	+ 30%	+ 25%	
Kraemer *et al.,* (1991)	8	Trained	8 exercises: 3/5 x 5 (5 RM)	+ 27%	+ 27 %	
Hakkinen & Pakarinen (1993)	10	Trained	1 exercise: 20 x 1 (100% 1 RM)	Nil		Nil
Kraemer et al., (1993)	9	Trained	8 exercises: 3/5 x 5 (5 RM)			Nil
Raastad *et al.,* (2000)	8	Trained	3 exercises: 3 x 3-6 (3-6 RM)	+ 17 %	- 7%	Nil
Smilios *et al.,* (2003)	11	Trained	4 exercises: 2 x 5 (88% 1 RM)	Nil		Nil
			4 exercises: 4 x 5 (88% 1 RM)	Nil		Nil
			4 exercises: 6 x 5 (88% 1 RM)	Nil		Nil
Zafeiridis *et al.,* (2003)	10	Trained	4 exercises: 4 x 5 (88% 1 RM)			Nil

(T = Testosterone, C = Cortisol)

Table 2.3 Summary of the acute hormonal responses to power protocol resistance exercise in males.

Author	Subjects (n)	Training Status	Protocol	Change in Hormone Concentration		
				T	IGF-1	C
Mero *et al.,* (1991)	9	Trained	1 exercise: 10 x 6 (50% 1 RM)	+ 18%		
Mero *et al.,* (1993)	6 (24y)	Trained	1 exercise: 10 x 6 (50% 1 RM) *	+ 16%		Nil
	6 (24y)	Trained	1 exercise: 10 x 6 (50% 1 RM) #	+ 18%		Nil
	6 (15y)	Trained	1 exercise: 10 x 6 (50% 1 RM) *	Nil		+ 67%
	6 (15y)	Trained	1 exercise: 10 x 6 (50% 1 RM) #	+ 13%		+33%
Volek *et al.,* (1997)	12	Trained	1 exercise: 5 x 10 (30% 1 RM)	+ 15 %		Nil
Pullinen *et al.,* (1998)	6 (25y)	Trained	1 exercise: 10 x 6 (50% 1 RM) *	+ 16%		
	6 (25y)	Trained	1 exercise: 10 x 6 (50% 1 RM) #	+ 18%		
	6 (15y)	Trained	1 exercise: 10 x 6 (50% 1 RM) *	Nil		
	6 (15 y)	Trained	1 exercise: 10 x 6 (50% 1 RM) #	+ 13%		

(T = Testosterone, C = Cortisol, * = 4 min rest period, # = 1 min rest period)

2.2.2 Sprint/Interval Exercise

Reports concerning the hormone response to interval exercise are few, particularly in relation to repeated sprint protocols. The responses to interval exercise are also difficult to compare given the multitude of exercise protocols that the terms sprint or interval exercise encompass. However, sprint and high intensity interval exercise have previously been shown to stimulate significant increases in concentrations of cortisol, total testosterone, prolactin, estradiol and GH to different protocols when performed in the form of both running, and cycling.

2.2.2.1 Sprint Running Interval Exercise

Sprint running interval repetitions of uniform, increasing and decreasing distance have been demonstrated to elicit changes in testosterone, cortisol and IGF-1 concentrations (Meckel *et al.,* 2009; Meckel *et al.,* 2011). In the first of these studies, with the aim to assess the influence of brief sprint interval exercise on the balance between anabolic (testosterone, IGF-1 and GH) and catabolic (cortisol) hormones, participants completed 4 x 250m running intervals on a treadmill at 80% of maximal speed, with 3 min recovery between each. Small but significant increases in testosterone were noted of 13%, while increases in IGF-1, although 16%, were not significant. Interestingly, following on from earlier discussion related to resistance exercise (Section 2.2.1), there were significant changes in IGF binding protein concentrations. Despite significant increases in lactate, cortisol concentration actually decreased resulting in a significant increase in the testosterone cortisol ratio. In using a similar subject group, Meckel *et al.* (2011) subsequently investigated the influence of increasing (100m - 400m) and decreasing (400m - 100m) sprint interval running repetitions, with increasing or decreasing rest respectively, on a treadmill on concentrations of IGF-1, testosterone and

cortisol. Similar results were demonstrated to the earlier study, with a significant increase in testosterone, accompanied this time by a significant increase in IGF-1 with no difference observed between trials in terms of concentrations, or area under the curve. No elevations in cortisol concentration was elicited. The similarity of results between the two studies may be expected given the same total session volume. The lack of cortisol response despite significant increases in lactate may be related to either long rest periods relative to exercise time, or the volume of the exercise.

Larger increases in hormone response have been observed in response to sprint running interval exercise where the total volume of the exercise session has been higher. Increases of 38%, 45%, 230% and 2000% in testosterone, estrogen, prolactin and GH, respectively have been observed in male athletes completing 16 x 1 min runs on a treadmill at the speed at which VO_2max was achieved, with 1 min walk between each (Gray *et al.,* 1993). The testosterone response to interval running exercise does appear to be linked to the lactate response and the intensity of the exercise completed. The studies above, implementing protocols considered to have a large anaerobic component with a significant proportion of time spent above lactate threshold have elicited a significant response, while no change in testosterone has been reported to 20 min runs at various submaximal exercise intensities (Galbo *et al.,* 1977; Wilkerson *et al.,* 1980). These contrasting results also raise the possibility that the form of exercise, continuous or intermittent, may also be important at modulating the hormone response observed to a given session with intermittent exercise seemingly more effective.

2.2.2.2 Sprint Cycling Interval Exercise

Despite the links stated above, relating intermittent exercise volume to hormone response, sprint cycling exercise of low volume has also been demonstrated to stimulate increases in circulating hormone concentrations. A single 6 s cycle sprint significantly elevated total testosterone concentration (Derbre *et al.,* 2010), with single 30 s cycling sprint stimulating increases in cortisol, testosterone, prolactin and GH concentrations (Stokes *et al.,* 2013), and single 30 s and 40 s cycle sprint demonstrating increases in salivary testosterone and cortisol concentrations (Crewther *et al.,* 2010a; Crewther *et al.*, 2010b). The findings of Derbre *et al.,* (2010) also imply a link to training status, with trained participants demonstrating an increase in testosterone to the single sprint, while untrained participants did not. However, trained participants were also able to complete a greater amount of work over the 6 s sprint which may also contribute to this observation. The study of Stokes *et al.* (2013) investigated the response to a single sprint of longer duration in recreationally active males. A significant in testosterone and cortosol was observed. It may be that the longer duration of the sprint was able to elicit a hormonal response in less trained individuals than those of the study of Derbre *et al.*, (2010). Crewther *et al.,* (2010a) utilised the same protocol and a similar subject group to Stokes *et al.* (2013) and demonstrated significant increases in salivary and venous testosterone and cortisol. Venous testosterone concentrations were elevated by 8% immediately following exercise, while salivary testosterone concentration was elevated 35% above resting levels 10 min post exercise. Venous concentrations of cortisol did not change in response to the sprint, but salivary testosterone was elevated by 63% at 20min post exercise. The same authors used 40s upper and lower body sprints in a later study, with a highly trained group of participants consisting of elite male rugby players (Crewther *et al.,* 2010b). The upper body cycle sprint did not

influence hormone concentrations, however, the lower body cycle sprint resulted in increases in salivary testosterone. Similar to observations relating to resistance exercise, this may suggest that active muscle mass is an important factor in determining the hormone response to exercise, an assertion that has been demonstrated previously, and thought to be linked to the metabolic stress generated (Lu *et al.,* 1997; Ratamess *et al.,* 2005). In addition, given the very limited volume of the protocols used that stimulate a hormone response, these findings suggest, similar to some assertions relating to sprint running interval exercise, that the intensity of the exercise is a key factor in stimulating hormonal responses.

In terms of repeated sprint/intermittent cycling exercise, free testosterone concentration have been shown to increase in response to a bout of repeated 5 s sprint (Goto *et al.,* 2005). Similarly to sprint running exercise, sprint cycling interval exercise does not appear to elicit an increase in all hormones with no response in IGF-1 observed following a 30 s cycle sprint (Stokes *et al.,* 2010). A lack of IGF-1 response to exercise has been associated with an increase in pro-inflammatory cytokines that often accompanies high intensity exercise (Bishop *et al.,* 2002) and may attenuate the IGF-1 response (Eliakim & Nemet, 2010).

2.2.3 Concurrent Training

Where two modes of exercise are trained concurrently an interference effect has been proposed (Docherty and Sporer, 2000) relating to the observed compromised strength gains when strength and endurance are trained concurrently (Bell *et al.*, 2000; Hakkinen *et al.*, 2003; Chtara *et al.*, 2008). The majority of research to have considered concurrent training has investigated the effect of strength and endurance training performed within the same session. Total testosterone concentration has been reported to be greater post-training following a training session where strength training of 3 x 8 repetitions at 75% 1RM of four exercises followed 30 min of aerobic cycling exercise at 75% of maximal heart (Cadore *et al.,* 2012), and also after 3 x 10 repetitions at 70% 1 RM of four exercises followed and intermittent running protocol (Rosa *et al.,* 2014). Post-exercise concentrations of cortisol did not differ depending on strength or endurance exercise order in either study, with the authors suggesting a training order of endurance exercise followed by strength exercise should be prescribed if the main focus of the training intervention is to induce an acute post-exercise anabolic environment. This assertion is supported further by research demonstrating greater increases in cortisol following performance of endurance exercise after strength exercise, that were also accompanied by increased neuromuscular fatigue, and decreased testosterone concentrations at 24 and 48 h post-exercise in comparison to performance of strength exercise after endurance exercise (Taipale & Hakkinen, 2013). The strength and endurance protocols used were slightly different to those in the aformentioned studies. The strength protocol consisted of 3 x 5 - 8 repetitions at 75 - 80% 1 RM followed by to 3 x 8 - 10 repetitions at 30-40% 1 RM at high velocity in a circuit that was comprised of four exercises. The endurance exercise consisted of 60mins of steady state running at lactate threshold. Despite these differences in protocol between studies, similar

responses have been observed and conclusions drawn. These findings are, however, of limited practical value in an elite sport setting as there are very limited occasions where, in that particular environment, athletes would perform sessions concurrently. It may therefore be surprising that limited research has been conducted into the influence of hormone responses to an exercise bout and the hormone response and performance of a subsequent bout. Within the limited research, contrasting findings have been presented. Performance of cycle sprint exercise performed 60 min or 180 min prior to a resistance exercise session, did not result in any differences being observed in the responses of cortisol and free testosterone to the resistance exercise bout in comparison to when in was performed alone (Goto *et al.*, 2007). Equally, completion of 60 min of endurance cycling at 50% VO_2max, did not influence the testosterone and cortisol response to subsequent resistance exercise (Goto *et al.,* 2005). In contrast, however, Ronsen *et al.* (2001) demonstrated a significantly greater increase in cortisol concentration following a repeated high intensity endurance exercise bout with 3 h recovery. Also in this study, pre-exercise concentrations of testosterone were lower prior to the second bout of exercise, but experienced a greater relative increase.

2.3 Effects of Acute Hormone Response on Performance

2.3.1 Strength & Power

Following compelling evidence demonstrating that purportedly anabolic hormones do not influence the gains in muscle strength and mass following resistance training (West et al., 2009; West et al, 2010; West & Phillips, 2010), recent research has investigated the ability of these hormones, in particular testosterone, to enhance acute performance of strength and power tasks.

Performance of a lower body cycle sprint prior to the performance of strength and power tasks resulted in an elevation in salivary testosterone. While this response was related to an improvement in the subsequent strength task, power output was not improved (Crewther *et al.,* 2011). However, significant relationships have been observed between resting saliva testosterone and cortisol concentrations and jump power and squatting strength (Crewther *et al.,* 2009a), with pre-exercise concentrations of free testosterone also having been demonstrated to be significantly related to 1 RM squat performance and 10 m sprint running time (Crewther *et al.,* 2012a). The same authors have also demonstrated further strong correlations between salivary concentrations of testosterone and cortisol and measures of speed, power and strength (Crewther *et al.,* 2009b; Cook & Crewther, 2010), and suggest the confirmation of a relationship between neuromuscular performance and hormone secretion patterns.

Evidence exists suggesting that the effect on performance of elevations in testosterone concentration may be sustained with Cook *et al.* (2013) observing improvements in jump peak power, 400 m sprint time and 3 RM bench and squat performance performed 6 h after a morning resistance exercise session that offset the circadian decrease in

testosterone concentration. In addition, increases in testosterone in response to different resistance exercise protocols performed as much as 3-4 days before a competitive rugby match have been shown to be associated with winning performances, with relative changes in testosterone in response to the resistance exercise bout significantly higher before matches resulting in a win (Crewther *et al.,* 2013). However, it is very unclear as to whether the hormonal changes observed in these two studies are causal of the improvements observed.

Recently, interest has been raised in the downstream androgen converted from testosterone, DHT. DHT is two to three times more potent than its precursor (Bauer *et al.,* 2000) and has also been suggested to exert both functional and signalling effects in skeletal muscle above those of testosterone. Indeed, in vitro, it has been shown that treatment of muscle fibres with physiological doses of DHT modulates muscle fibres in a fibre-type dependent manner, increasing power in fast twitch fibres, while treatment with testosterone did not (Hamdi & Mutungi, 2010).

2.3.2 Mood & Behaviour

Following the evidence presented in section 2.1.2.3 regarding the ability of testosterone to influence mood, behaviour and cognitive function, recent research has sought to assess the influence of using short video clips in a pre-workout environment on change in hormone concentrations, athlete behaviour and subsequent voluntary performance (Cook & Crewther, 2012a; Cook & Crewther, 2012b).

Highly trained athletes were exposed to a randomised presentation of short video clips 15 min prior to the start of a workout. Significant relative increases in salivary

testosterone were measured following the watching of erotic, humorous, aggressive and training videos in comparison to a control. 3 RM performance was improved following viewing of the erotic, aggressive and training video clips. A strong within individual correlation was also noted between changes in testosterone and 3 RM performance across all video clips (Cook & Crewther, 2012a).

In a subsequent study, the same authors investigated the influence of pre-match motivational interventions on the free testosterone and cortisol concentrations and subsequent match performance in rugby. Watching a video clip of successful skill performance along with positive feedback from the coach elevated testosterone concentrations beyond that of self-motivational practices or the viewing of video clips with cautionary feedback which elicited a elevation of cortisol. Across all trials, greater testosterone responses and lower cortisol responses were associated with enhanced motivational behaviour and performance of identified skills (Cook & Crewther, 2012b).

Similar observation have been made in sports such as judo were positive associations have been demonstrated between free testosterone concentrations and offensive behaviours, with additional links identified between higher testosterone concentrations in winners of both physical and non-physical tasks (Kilduff *et al.,* 2013). Combined, these studies support a role for testosterone and cortisol in enhancing acute strength and skill performance that is associated with motivation and confidence to compete.

- Chapter 3 -

General Methods

3.1 Introduction

This chapter describes the methodologies implemented during the studies comprising this thesis. Each of the investigations was carried out in the Exercise Physiology Laboratories at the University of Bath, and was approved by either the Local NHS Research Ethics Committee or the University of Bath Research Ethics Approval Committee for Health prior to commencing. The target populations for the investigations were male and female non-smokers, aged between 18 and 35 years who, depending on the study, were highly trained in either cycling, resistance exercise, or had no specific training history and were less well trained. Recruitment was done via advertisements around the university campus and training facilities, as well as through local sports clubs. After volunteering, participants were fully briefed as to the requirements of the investigation both verbally and in writing. Volunteers who felt they could fulfil the requirements of the investigation were then asked to provide written informed consent. Compulsory health screen questionnaires were completed by participants prior to commencing any testing, and participants were withdrawn from the investigation if any medical condition that posed a personal risk was reported.

3.2 Anthropometry

Body mass was measured as part of each participant's initial visit to the laboratory. Measurements were made using a balance scale (Avery Ltd, UK) were accurate to ± 0.5 kg. Body mass was recorded prior to any exercise training session throughout each of the studies.

Height was measured using a wall mounted stadiometer (Holtain Ltd, UK) that had a maximum range of 210 cm and was accurate to ± 0.01 cm. Height was measured without footwear, with the heels together and the participant looking straight ahead. The moveable gauge was lowered until contact with the participant's head while they inhaled deeply.

3.3 Preliminary Measurements

Prior to experimental trials, participants were required to visit the laboratory on up to two occasions. During these visits, anthropometric measures were taken as described above and exercise tests relevant to the investigation were completed in order to assess each participant's maximal performance characteristics.

3.3.1 Maximal Oxygen Uptake

During the investigations described in Chapters 4, 5, 7 and 8, participants completed a test to determine maximal oxygen uptake (VO_2max), and the cycling power output associated with this value. The test involved continuous, incremental cycling on a stationary ergometer until the point of volitional exhaustion. Participants completed a 10 min warm up at a self-selected intensity. The starting resistance of the test was adjusted for each participant in order that the test last approximately 12 – 15 min, and was increased by 30 W every 3 min. In the final minute of each stage, heart rate, ratings of perceived exertion and one minute expired air samples were collected. These measures were also obtained at the point of volitional exhaustion. A true reflection of VO_2max was considered to have been attained where the respiratory exchange ratio (RER) recorded was in excess of 1.15, and an increase in VO_2 ≤ 5 $ml.kg^{-1}.min^{-1}$ in response to an increase in resistance from the previous stage was observed.

3.3.2 Maximal Strength Testing

Participants completing the investigations described in Chapters 4, 5, 7 and 10 completed tests to determine maximal strength. This test was performed in the laboratory on either a Concept 2 dynamometer (Concept 2, Notts, UK), or on a Keiser A420 Leg Press (Keiser Ltd, Tetbury, UK).

3.3.2.1 Concept II Dynamometer Test: The Concept II dynamometer is a multi-purpose resistance machine providing a measure of force production (presented in kg) based on the acceleration applied to an internal flywheel. The force required for acceleration can be calculated since the moment of inertia of the fan is a known constant. The component of the force needed to resist the air drag is a function of the speed of the wheel and the air drag coefficient, which is determined during each run-down of the fan.

Participants completed a test to determine their maximal strength for the exercises of leg press, chest press and upright bench pull on a Concept 2 dynamometer (Concept II, Notts, UK). Each lift was performed after the five second countdown indicated on the display unit, in order to allow the flywheel to settle. Participants warmed up on each lift at a self-selected intensity before the test started. Before the dynamometer will record data, three warm up, or pre-, lifts are required to be performed. Participants completed these three pre-lifts before performing three repetitions at maximum effort, leaving the required five seconds between repetitions. Participants completed the test first on chest press, followed by leg press, and finally upright bench pull.

3.3.2.2 Leg Press Test: Participants completed an incremental test to determine their one repetition maximum for leg press on a Keiser A420 leg press (Keiser Ltd, Tetbury, UK). The leg press is designed to produce consistent resistance across the entire range of movement via the use of air pressure, and is designed to produce accurate position, velocity, power and acceleration information throughout the range of movement. The equipment was calibrated bimonthly in a procedure that involved three steps: setting the zero point of the pressure transducers, setting the zero point of the position transducers, and setting the span of the position transducers. To set the zero for the

pressure transducers, the machine is reduced to minimum resistance before all air hoses are disconnected from both machine and compressor. The pressure is then set to zero in the software. The air hoses are then reconnected before setting the zero point for the position transducers by moving the foot plates through range to their outer stops and setting the zero point in the software once again. The span of the position transducers is calibrated using a calibration tool with two different length calibration blocks scored into it. For each of the moving arms of the machine, first, the shorter of the two calibration block is inserted between the collar at the end of moving part of the cylinder and the bolt at the end of the piston before setting the position point in the software. The same process is then repeated with the longer calibration block. The machine is then re-calibrated for pressure and position. In order to determine maximal strength, participants completed the ten repetition test protocol programme installed on the leg press, completing single repetitions at progressively higher loads starting at a low relative percentage of predicted one repetition maximum. The test continued until the participant could not complete a lift. One repetition maximum was determined as the highest weight at which one full repetition was completed with the correct technique.

3.3.2.3 10 s Maximum Cycle Sprint Test: Participants completed a 10 s maximum cycle sprint test to determine maximum sprint power on a Wattbike stationary cycle ergometer (Wattbike Ltd, Notts, UK). The Wattbike is factory calibrated and does not need recalibrating. It measures absolute mechanical power in Watts with the amount of power produced measured from the sum of all the forces applied to the chain through the cranks. The forces are measured by one load cell and sequencing of the applied force is calculated according to crank position which is determined by the location of two magnetic sensors on the crank. Prior to completing the 10 s test in order to

determine peak power, participants warmed up at a self-selected intensity for 5 - 10 minutes during which time they trialled different resistances to find an appropriate starting gear. Following the warm up, participants completed a maximum 10 s effort, in their self-selected gear, from a standing start. Maximum and average power output, and peak and average cadence were recorded.

3.4 Physiological Measurements

3.4.1 Expired Gas Analysis

Prior to any expired gas collection, participants were provided with a nose clip and respiratory valve 30 – 45 s beforehand in order to flush all atmospheric air from the apparatus prior to the sample being taken. Falconia tubing was attached to the respiratory valve and connected the valve to a Douglas bag for collection of expired gases. The relative fractions of oxygen and carbon dioxide were measured (Servomex 1440/5200, Servomex, UK). The analysers were calibrated prior to each test with a two point calibration using gas concentrations of known composition. The zero point was established with nitrogen (100%), and the upper limit with an O_2 - CO_2 mix (16% and 5% respectively). The analyser was then validated against atmospheric air. Expired gas volumes were determined during evacuation of each Douglas bag using a dry gas meter (Harvard Apparatus, UK). The temperature of the expired gases was recorded during evacuation using a thermistor probe. Rates of oxygen (VO_2) utilisation were then calculated with the use of a prepared spreadsheet.

3.4.2 Saliva Sampling

Samples were expressed via passive drool into 15 ml plastic collection tubes with no additive. Participants were asked to refrain from eating, drinking, or brushing teeth drinking in the 1 h prior to sample collection, rinse their mouth with water 5 min prior to collection (Shirtcliff *et al.*, 2002), and remain seated while expressing the sample. Samples were dispensed into separate eppendorfs and frozen at -20°C until analysis. For analysis, samples were defrosted at room temperature, and subsequently centrifuged at 3000 rpm for 10 mins. Clear supernatants were then assayed for concentrations of testosterone and cortisol. Flow rate was not measured or corrected for as concentration

of unconjugated steroids such as testosterone and cortisol is not influenced by flow rate (Vining *et al*., 1983).

3.4.3 Saliva Analysis

Commercially available enzyme linked immunoassays (ELISA) (Salimetrics Europe, Oxford, UK) were used to analyse saliva for testosterone and cortisol. The assays are competitive ELISAs and operate on the principle that the hormone in the sample competes with the hormone linked to horseradish peroxidise for the antibody binding sites. Following incubation, the unbound components are washed away, and the bound amount of the hormone linked to peroxidase is measured by the reaction of the peroxidise enzyme on the substrate tetramethylbenzidine (TMB) which produces a blue colour. Upon stopping the reaction with sulphuric acid a yellow colour is formed. The optical density was then measured by the assay plate being read on a standard plate reader (Anthos II, Anthos, Krefeld, Germany) at 450 nm. The amount of hormone linked to peroxidase detected is inversely proportional to the concentration of the hormone present in the sample.

3.4.4 Blood Sampling

3.4.4.1 Venous Blood Sampling: Blood samples were drawn via venepuncture from an anterior antecubital vein. Participants were required to lie on a medical bench during sampling. At each sampling time-point a 10 ml whole blood sample was drawn. Of this, 5 ml was dispensed into a blood collection tube (Sarstedt Ltd., UK) containing the anticoagulant ethylenediaminetetraacetic acid (EDTA). This sample was immediately analysed for concentrations of lactate prior to being centrifuged at 1500 g for 10 min. Plasma was subsequently removed from the sample and aliquoted into separate

individually labelled eppendorfs before being frozen at -20°C until analysis. The remaining 5 ml was transferred into a blood collection tube containing no anticoagulant (Sarstedt Ltd., UK) and was left to clot for ~15 min at room temperature and then centrifuged at 1500 g for 10 mins before the serum was removed, dispensed into separate individually labelled eppendorfs and frozen at -20°C until analysis.

3.4.4.2 Capillary Blood Sampling: Blood samples were drawn from the finger tip following using a lancet device to make a small pin-prick incision. Participants were seated for sampling. At each sampling time-point two 500 ul samples of whole blood were drawn. One sample was collected into a blood collection tube containing the anti-coagulant EDTA (Sarstedt Ltd., UK), and centrifuged immediately in a microcentrifuge at 10 000 rpm for 5 min. Plasma was removed from the sample, transferred to labelled eppendorfs and frozen at -20oC until analysis. The other sample was collected into a blood collection tube containing no anticoagulant (Sarstedt Ltd., UK) and was left to clot for ~15min before being centrifuged in a microcentrifuge at 10 000 rpm for 5 min. Serum was removed from the sample, transferred to labelled eppendorfs and frozen at -20°C until analysis.

3.4.5 Blood Analysis

3.4.5.1 Total Testosterone

Serum analysed for total testosterone in Chapters 4, 5 and 7 was done by liquid chromatography tandem mass spectrometry (LC-MS-MS). During this procedure, deuterium labelled internal standard is added to the sample, and the testosterone in the sample is then isolated by liquid liquid extraction. The extract is then dried under a stream of nitrogen. For determination of testosterone, the sample extract is reconstituted

and quantified using multiple reaction monitoring of testosterone relative to the internal standard. There are no interferences from common steroids found in human serum (Fitzgerald *et al.,* 2010).

Serum analysed for total testosterone in Chapters 8, 9 and 10 was done by ELISA using commercially available kits (IBL, Hamburg, Germany). The procedure and principles are the same as those stated above in section 3.4.3.

3.4.5.2 Cortisol

Serum concentrations of cortisol in Chapters 4, 5, and 7 were determined by automated, solid-phase, competitive chemiluminescent enzyme immunoassay (Immulite®). Serum analysed for concentrations cortisol during Chapters 8, 9 and 10 was done so using commercially available kits (IBL, Hamburg, Germany). The procedure and principles are the same as those stated above in section 3.4.3.

3.4.5.3 Free Testosterone & DHT

Serum analysed for concentrations of free testosterone, DHT and cortisol during Chapters 8, 9 and 10 was done so using commercially available kits (IBL, Hamburg, Germany). The assay principles are the same as those stated above in section 3.4.3.

3.4.5.4 Total IGF-1 & Free IGF-1

During Chapter 4, plasma was analysed for total IGF-1 using a commercially available kit (Active IGF-I ELISA, DSL-10-5600), involving a simple acid-ethanol extraction procedure in which IGF-I was separated from its binding proteins. Determination of plasma free-IGF-I concentrations was carried out using a commercially available kit

(Active free IGF-I ELISA, DSL-10-9400) (DSL Europe, Oxford, UK). The assay principles are the same as those stated above in section 3.4.3.

3.4.5.5 Prolactin, Estradiol, Progesterone, FSH & LH

Serum analysed for concentrations of prolactin, estradiol, progesterone, FSH and LH was carried out using a semi-automated biochip immunoassay analyser (Evidence Investgator, Randox Laboratories, Co. Antrim, UK).

Table 3.1 Intra- and inter-assay coefficient of variation (CV) for all hormones measured.

Measure	Intra-Assay CV (%)	Inter-Assay CV (%)
Saliva:		
Testosterone	4.6	5.7
Cortisol	3.5	5.1
Blood (Serum):		
Testosterone		
Free Testosterone	8.9	8.8
DHT	6.9	8.5
Cortisol	3.0	3.5
Prolactin	8.0	
Progesterone	10.3	
Estradiol	8.5	
LH	5.7	
FSH	6.3	
Blood (Plasma):		
IGF-1	6.0	6.7
Free IGF-1	3.1	9.1

3.5 Subjective Data

3.5.1 Ratings of Perceived Exertion

Sensations of fatigue and exertion were monitored during maximal oxygen uptake tests in Chapters 4, 5, 7, and 8, and throughout trials in Chapter 9 via a rating of perceived exertion (RPE) scale as described by Borg (1973). The RPE scale is graded from 6-20, with these numbers reflective of perceptions of effort ranging from 7 being 'Very, very light' to 20 which corresponds to 'Maximum' effort.

3.5.2 Psychological Affect

Psychological affect (Chapter 9) was assessed using the Feeling Scale (Hardy and Rejeski, 1989). This is an 11-point bipolar measure of pleasure-displeasure, ranging from +5 (I feel very good) to -5 (I feel very bad).

3.6 Standardisation Procedures

3.6.1 Ambient Conditions

Ambient temperature, pressure and humidity were monitored throughout each trial and controlled where possible in order to ensure that, as far as possible, main trials were performed under the same environmental conditions.

3.6.2 Dietary Control

In order to ensure participants reported for each trial in the same nutritional state, each participant was provided with a dietary record sheet on which they were asked to record all food and drink intake over the 24 h prior to their first main trial. This diet was then adhered to in the 24 h before each subsequent trial. Participants were asked to avoid consumption of alcohol and caffeine in the 24 h leading up to each main trial.

3.6.3 Habitual Training

Participants were permitted to continue with their habitual training during their period of participation in any trial. In the 48 h prior to each main trial, participants were asked to avoid high intensity exercise, and to repeat their activity patterns as far as was practicable.

3.7 Statistical Analysis

3.7.1 Bland Altman Limits of Agreement

Bland Altman limits of agreement (LOA) analysis was used to assess agreement between sampling methods in Chapters 4 and 5. Firstly, the mean difference (bias) between the two measures was established, along with the standard deviation. Limits of agreement were then computed using mean ± 2SD. Ratio LOA were calculated using the same procedures following log transformation, and subsequent back transformation, of the data.

3.7.2 ANOVA

One-way repeated measures analysis of variance (ANOVA) was used to analyse variables measured once per trial. A two way repeated measures ANOVA was used to analyse variables measured through time. Greenhouse-Geisser epsilon corrections were used when the sphericity assumption was violated, whilst the Huynh-Feldt correction was utilised for less severe asphericity (Atkinson, 2001). Significant main effects were investigated further using multiple paired t-tests to determine the location of variance, post hoc Bonferroni correction was then used to adjust for the number of pairwise comparisons completed.

3.7.3 T-Tests and Correlations

In Chapters 4 and 5, paired two tailed T-tests were also used independently to compare the means between two different groups. Significance was accepted at $P < 0.05$. Bonferroni correction was applied where multiple t-tests were completed.

Correlations were used during all studies, with associations between variables assessed using Pearson's correlation or Spearman's Rank test. The strength of correlations observed was interpreted according to Cohen's effect sizes (Cohen, 1988) and is as follows:

Table 3.2 Interpretation criteria for observed correlation coefficients

r	Effect Size	Correlation
0.1	Small	Weak
0.3	Medium	Moderate
0.5	Large	Strong

3.7.4 Post-Hoc Power Calculations

Post-hoc power calculations were performed where near significant results were found in order to establish sample size required to achieve significance. Calculations were performed using G*Power software version 3.1.9.2 (Dusseldorf, Germany). Alpha was set to 0.05, beta to 0.95, and observed means and standard deviations values were entered to calculate observed power, from which sample size to achieve desired power was calculated.

- Chapter 4 -

Agreement between Venous and Capillary Hormone Concentrations at Rest, and in Response to Resistance and Sprint Exercise

4.1 Introduction

Concentrations of a number of hormones have been suggested to be important in athletic performance and adaptation to exercise training, as well as to providing an indication of the physiological state of athletes (Nindl *et al*., 2003; Kraemer & Ratamess, 2005; Beaven *et al.,* 2008). Circulating concentrations of some of these hormones are also known to change in response to exercise stimuli (Kraemer *et al*., 1990), and have also been demonstrated to change in response to different nutritional practices with manipulation of dietary protein and fat intake (Crewther *et al.,* 2006; Kraemer & Ratamess, 2005; Volek, 2004; Volek *et al.,* 1997). Despite the debate relating to whether such responses to exercise influence training adaptation (Kraemer & Ratamess, 2005; Spiering *et al*., 2008; West *et al*., 2010; Wilkinson *et al*., 2006), these hormones are regularly monitored in elite sport.

The traditional reference method for measuring hormone concentrations has been venous blood; but this can be impractical in some applied training situations. Capillary blood collection methods are accepted as more convenient and less invasive methods that enable more frequent measurement of hormone concentrations outside of a laboratory setting.

The relationship between venous and capillary concentrations of growth hormone is reported to be strong (Godfrey *et al*., 2004), and agreement between venous and capillary concentrations of plasma cortisol has recently been shown during high intensity exercise (Fryer *et al*. 2012). Nindl *et al*., (2003) have previously compared IGF-1 concentration obtained from a filter paper capillary blood spot assay with venous blood concentrations demonstrating a strong significant correlation (r = .920), and the ability of the blood spot concentrations to predict serum values. However, measured blood spot concentrations were on average 61% lower than serum values. No previous research has investigated the validity of capillary blood sampling for the measurement of circulating IGF-1 concentrations using standard ELISA methods.

Indeed, given the possible suggested role of hormones in adaptation to exercise training, in particular IGF-1, cortisol and the sex steroids such as testosterone very little research has considered the agreement between venous and capillary hormone concentrations. Even less research has been conducted into the agreement between methods following intense exercise. The present research endeavoured to address this by including a wide battery of hormones for analysis and assessing agreement between methods at rest and in response to both resistance and high intensity interval exercise. Specifically, in order to establish whether capillary methods can be used as an acceptable alternative to venous sampling, the purpose of this investigation was to assess, at rest and following exercise, the agreement between venous and capillary samples for concentrations of total cortisol, total testosterone, free testosterone, total IGF-1, free IGF-1, total estradiol, total progesterone, total prolactin, total FSH, and total LH.

4.2 Methods

4.2.1 Subjects

Eleven physically active men (age 28 ± 3 y; body mass (BM) 77.5 ± 8.3 kg; $\dot{V}O_2$ max 57.2 ± 9.0 ml·kg $BM^{-1}·min^{-1}$) provided written informed consent before participating in the study, which was approved by the Bath Local National Health Service Research Ethics Committee. Participants had a range of sporting backgrounds, and habitual training varied from light (2-3 sessions per week) to substantial (8-10 sessions per week).

4.2.2 Preliminary Measurements

Preliminary tests were conducted in order to determine each participant's maximal oxygen uptake. Participants completed an incremental test to exhaustion on a SRM cycle ergometer (Schoberer Rad Messtechnik, Fuchsend, Germany). The protocol included a 10 min warm-up at self-selected intensities followed by consecutive 3 min stages, at the end of which the load on the ergometer was increased by 30 W. In the final minute of each stage, a one minute expired air sample was collected and analysed for concentrations of O_2 and CO_2 to assess oxygen uptake. Three maximal repetitions on each resistance exercise of leg press, seated bench press and seated bench pull were then also completed on the concept 2 dynamometer (Concept2 DYNO, Notts, UK). Data were used to calculate work intensities for the subsequent experimental trials. All participants continued their habitual training throughout the study period but were asked to refrain from strenuous exercise and avoid both alcohol and caffeine consumption during the 24 h prior to any main trial.

4.2.3 Experimental Design

Participants completed up to three trials in a randomised order separated by 5 - 10 days and were randomly allocated into two groups. One group completed two trials consisting of two exercise bouts (a cycling sprint interval exercise bout and a resistance exercise bout) separated by 5 h. The sprint interval exercise was performed on an SRM ergometer and comprised a 10 min warm up followed by 10 x 30 s sprints with 90 s recovery between each sprint. Participants were asked to aim for a peak power of 150 % power at $\dot{V}O_{2\,max}$ and then try to sustain a power output as close to that as they could for the remainder of each 30-s sprint. The resistance exercise session consisted of 5 sets of 10 repetitions at 80% of maximum on seated bench press, leg press and seated bench pull exercises. Exercises were performed as a superset (no rest interval between exercises) in the aforementioned order, with one minute recovery after each superset. In one trial, participants performed the interval exercise session in the morning and the resistance session in the afternoon, and in the other trial they performed the sessions in the reverse order. The second group completed three trials: a rest day; a rest day with carbohydrate-protein supplementation in addition to their normal diet; and a day on which they performed cycling sprint interval exercise. Exercise and protein intake have previously been shown to influence hormone concentrations (Crewther, *et al.,* 2006; Kraemer & Ratamess, 2005; Volek, 2004; Volek, *et al.,* 1997).

4.2.4 Experimental Protocol

Participants arrived in the laboratory at the same time of day for each trial. They continued their habitual training throughout the study period but refrained from strenuous exercise and avoided alcohol and caffeine consumption in the 24 h prior to main trials. Venous and capillary samples were obtained on arrival at the laboratory.

Participants in the first group completed a bout of exercise (sprint interval exercise or resistance exercise) after baseline sampling, and then performed the other exercise bout (either sprint or resistance) 5 h later. Samples were taken immediately and 1 h after each exercise bout. In the second group, during the rest trial, further samples were obtained at 2, 4 and 6 h. In the protein trial, participants consumed a carbohydrate-protein drink (carbohydrate and whey protein isolate; 1:1 ratio; 0.5 $g{\cdot}kg^{-1}$ protein) immediately after baseline sampling, and further samples were obtained at 1, 2, 4 and 6 h. Participants consumed an additional 0.17 $g{\cdot}kg^{-1}$ protein following sampling at 1, 2 and 4 h to provide a total of 1.0 $g{\cdot}kg^{-1}$ protein supplementary to their diet. In the cycling interval trial, exercise was performed immediately after baseline sampling, and subsequent samples were obtained immediately post exercise and at 1, 2 and 4 h post-exercise.

4.2.5 Sampling and Analysis

Capillary blood samples were taken from the finger tip and collected in serum and EDTA microvettes (Sarstedt, UK). Venous blood samples were taken from a superficial antecubital vein. Serum samples were left to clot for 15 min, centrifuged and serum was stored at -20°C until analysis. Plasma samples were centrifuges immediately and plasma was stored at -20°C until analysis.

Serum was analysed for total cortisol, total testosterone, total IGF-1, free IGF-1, total estradiol, total progesterone, total prolactin, total FSH and total LH concentrations. Serum total testosterone concentrations were determined via Isotope Dilution Liquid Chromatography Tandem Mass Spectrometry (LC-MS-MS). Quantitation of results was performed by an internal standard method using Analyst® software version 1.4. Reporting range for the assay was between 0.7 - 60.0 $nmol{\cdot}l^{-1}$. Serum concentrations of cortisol were determined by solid-phase, competitive chemiluminescent enzyme

immunoassay (Immulite®). Serum concentrations of total estradiol, total progesterone, total prolactin, total FSH and total LH were determined via a semi-automated biochip immunoassay analyser (Evidence Investgator, Randox Laboratories, Co. Antrim, UK). Intra assay CV of the fertility array was 8.5, 10.3, 8.0, 6.3, and 5.7% for estradiol, progesterone, prolactin, FSH and LH respectively. Analysis methods for the different media were selected based upon being considered valid and established measurement methods.

Plasma was analysed for total IGF-1 using a commercially available kit (Active IGF-I ELISA, DSL-10-5600), involving a simple acid-ethanol extraction procedure in which IGF-I was separated from its binding proteins, with an assay sensitivity of 0.004 nmol.l^{-1}. Determination of plasma free-IGF-I concentrations was carried out using a commercially available kit (Active free IGF-I ELISA, DSL-10-9400), with an assay sensitivity of 0.002 nmol.l^{-1} (DSL Europe, Oxford, UK). Intra-assay CVs for total and free IGF-1 were 6.0 and 3.1% respectively; and inter-assay CVs were 6.7 and 9.1% for total and free IGF-1 respectively.

4.2.6 Statistical Analysis

Normality tests revealed non-normal distribution of all data. As such, Spearman correlations were used to identify the relation between concentrations from different samples. Bland-Altman analyses were used to determine 95% limits of agreement (LOA) between capillary samples with their venous sample equivalent. As some degree of heteroscedasticity was evident in all Bland-Altman difference plots, log transformation and subsequent back transformation of the data was used to establish LOA, with limits also expressed as ratio LOA (Atkinson & Nevill, 1998; Bland &

Altman, 2007). Paired t-tests were used to determine the presence of significant differences between sample types for each hormone at rest, following exercise, and with all samples combined. Data are presented as means and standard deviations with significance accepted at an alpha level of $P \leq 0.05$.

4.3 Results

Hormone concentrations measured following carbohydrate-protein supplementation were not different from those measured during the rest trial, and these data were therefore combined for analysis. A robust response in cortisol, testosterone, progestrone, estradiol and prolactin concentrations occurred in response to interval exercise, with a smaller response in total cortisol, testosterone, progesterone and estradiol in response to resistance exercise. Total and free IGF-1 concentration did not change in response to either interval or resistance exercise, and the response of FSH and LH to both forms of exercise was unpredictable.

Correlation between venous and capillary concentrations of total cortisol was very strong at rest (r = 0.907) and following exercise (r = 0.960; Table 4.1). In comparison to the range of concentrations measured, bias was small (-8.8 – -7.0 $nmol \cdot l^{-1}$), with venous concentrations measuring lower than capillary concentrations. LOA were similar at rest and following exercise and 95% ratio LOA for all samples suggest venous total cortisol concentrations to be between 16% lower and 13% greater than capillary concentrations.

For total testosterone, correlation was strong (r = 0.841), bias small (1.7 $nmol \cdot l^{-1}$) and LOA close (-6.2 – 10.3 $nmol \cdot l^{-1}$) when all samples were considered together, and were similar for samples obtained at rest and following exercise (Table 1). Venous total testosterone concentrations were significantly higher than capillary concentrations ($P < 0.01$). 95% ratio LOA suggest venous total testosterone concentration to be between 7% lower and 34% greater than capillary concentration.

Correlation between venous and capillary concentrations of total IGF-1 was moderate when all samples were combined (r = 0.472), at was slightly stronger at rest (r = 0.567) than following exercise (r = 0.412) There was a significant bias with capillary plasma concentrations higher than venous plasma concentrations ($P < 0.05$), although this was reduced following exercise (-8.6 nmol.l^{-1}) than at rest (-14.0 nmol.l^{-1}) The moderate correlations were accompanied by wide absolute LOA (Table 4.1). Limits of agreement were wider following exercise and 95% ratio LOA for all samples suggest capillary total IGF-1 concentration to be between 23% greater and 16% lower than venous concentration.

Correlation between venous and capillary free IGF-1 concentrations was strong for resting samples (r = 0.717) and slightly weaker post-exercise (r = 0.598). There was a significant bias with capillary plasma concentration measuring higher than venous plasma concentrations ($P < 0.01$) (Table 4.1). LOA were similar at rest and following exercise with 95% ratio LOA for all samples suggesting capillary free IGF-1 concentration to be between 52% greater and 40% lower than venous concentration.

For progesterone, correlation between venous and capillary concentrations was strong when all samples were considered together (r = 0.791) and at rest (r = 0.741), and spost-exercise samples (r = 0.860). Bias was small in comparison to the range of concentrations measured (0.87 - 48.3 nmol.l^{-1}), but was significant in resting samples (1.79 nmol.l^{-1}; $P < 0.01$). 95% ratio LOA suggest venous progesterone concentration to be between 67% greater and 24% lower than capillary concentrations.

Correlation between venous and capillary concentrations of estradiol was strong when considering all samples together (r = 0.764), at rest (r = 0.681) and following exercise (r = 0.823). Bias was small following exercise (0.005 $nmol.l^{-1}$), but greater at rest (0.011 $nmol.l^{-1}$; $P < 0.01$). 95% ratio LOA suggest venous estradiol concentrations to be between 12% lower and 33% greater than capillary concentrations.

For prolactin, correlation between venous and capillary concentrations was strong in both resting samples (r = 0.921) and those following exercise (r = 0.976). In relation to the range of concentrations measured (0.145 - 1.658 $nmol.l^{-1}$), bias was small but still significant in both resting samples (-0.015 $nmol.l^{-1}$; $P < 0.05$) and following exercise (-0.017 $nmol.l^{-1}$; $P < 0.05$) with capillary concentration measuring higher than venous concentrations. 95% ratio LOA indicate venous prolactin concentrations to be between 13% lower and 10% greater than capillary concentrations.

Correlation between venous and capillary concentrations of FSH were strong in both resting samples (r = 0.859), and in those following exercise (r = 0.858). Bias was small and non-significant in all cases with venous concentrations measuring higher than capillary concentrations. 95% ratio limits of agreement suggest venous concentrations of FSH to be between 12% lower and 18% greater than capillary concentrations.

LH demonstrated similar results to FSH. Correlation between venous and capillary samples was strong in both resting (r = 0.841) and post-exercise (r = 0.875). Bias was minimal, particularly following exercise (0.021 $IU.l^{-1}$) with venous concentrations measuring higher than capillary counterparts. 95% ratio LOA suggest venous LH

concentration to be between 15 % lower and 21% greater than capillary LH concentration.

Table 4.1 Correlation and LOA between venous and capillary blood samples for all samples, rest, exercise. (n = number of samples; Bias = venous concentration – capillary concentration).

Hormone	Condition	n	Range (nmol.l^{-1})	Correlation	Bias (nmol.l^{-1})	LOA (nmol.l^{-1})	Ratio LOA
Cortisol	All	119	99.0 - 807.0	.949**	-7.9	-102.3 - 86.5	0.84 - 1.13
	Rest	62	106.0 - 532.0	.907**	-8.8	-88.8 - 71.2	0.83 - 1.13
	Exercise	57	99.0 - 807.0	.960**	-7.0	-115.7 - 101.7	0.84 - 1.13
Total Testosterone	All	92	6.1 - 42.9	.841**	1.7*	-6.2 - 10.3	0.93 - 1.34
	Rest	44	7.8 - 33.2	.800**	2.7**	-5.2 - 10.5	0.95 - 1.40
	Exercise	48	6.1 - 42.9	.846**	1.1*	-7.1 - 9.9	0.91 - 1.28
Total IGF-1	All	83	161.6 - 538.0	.472**	-18.5*	-127.2 - 115.6	0.77 - 1.16
	Rest	28	161.6 - 476.7	.567**	-14.0	-114.3 - 86.3	0.82 - 1.14
	Exercise	55	196.5 - 538.0	.412**	-8.61*	-149.1 - 131.9	0.75 - 1.17

Free IGF-1	All	83	0.19 - 3.32	.611**	0.82**	-1.3 - 0.7	0.48 - 1.40
	Rest	28	0.24 - 2.79	.717**	0.80**	-1.3 - 0.7	0.47 - 1.34
	Exercise	55	0.19 - 3.32	.568**	0.84**	-1.4 - 0.8	0.49 - 1.44
Progesterone	All	140	0.87 - 48.28	.791**	1.49**	-8.4 - 11.4	0.76 - 1.67
	Rest	78	0.97 - 34.23	.741**	1.79**	-7.3 - 10.9	0.75 - 1.74
	Exercise	60	0.87 - 48.28	.860**	1.12	-9.8 - 12.1	0.78 - 1.57
Estradiol	All	140	0.052 - 0.202	.764**	0.008**	-0.039 - 0.056	0.88 - 1.33
	Rest	78	0.054 - 0.193	.681**	0.011**	-0.039 - 0.061	0.90 - 1.38
	Exercise	60	0.052 - 0.202	.823**	0.005	-0.039 - 0.049	0.87 - 1.28
Prolactin	All	144	0.145 - 1.659	.951**	-0.016**	-0.136 - 0.106	0.87 - 1.10
	Rest	80	0.165 - 1.054	.921**	-0.015*	-0.125 - 0.094	0.86 - 1.10
	Exercise	64	0.145 - 1.659	.976**	-0.017*	-0.149 - 0.115	0.91 - 1.15

Hormone	Condition	n	Range ($IU.l^{-1}$)	Correlation	Bias ($IU.l^{-1}$)	LOA ($IU.l^{-1}$)	Ratio LOA
FSH	All	144	0.64 - 5.93	.868**	0.087	-1.0 - 1.1	0.88 - 1.18
	Rest	80	0.77 - 5.93	.859**	0.076	-1.1 - 1.3	0.86 - 1.19
	Exercise	64	0.64 - 5.39	.858**	0.101	-0.7 - 0.9	0.91 - 1.16
LH	All	140	1.2 - 9.4	.858**	0.066	-1.3 - 1.5	0.85 - 1.21
	Rest	78	1.2 - 8.2	.841**	0.100	-1.5 - 1.7	0.85 - 1.24
	Exercise	60	1.4 - 9.4	.875**	0.021	-1.1 - 1.1	0.87 - 1.16

*$P < 0.05$, **$P < 0.01$

4.4 Discussion

This is the first study to provide a comprehensive report on the agreement between venous and capillary concentrations over a wide array of hormones. While the agreement between venous and capillary cortisol concentrations has previously been shown (Fryer *et al.*, 2013), this is the first demonstration that there is good relation and agreement between venous and capillary concentrations of total testosterone, progesterone, estradiol, prolactin, FSH and LH; suggesting that capillary sampling may be used as an acceptable alternative to venous sampling for these measures. Correlation and agreement between venous concentrations of total and free IGF-1 and capillary equivalents was only moderate, suggesting that venous and capillary blood sampling may not be used as an alternative to venous sampling for determination of total and free IGF-1.

Strong correlation and narrow LOA between venous and capillary concentrations of total cortisol, total testosterone, progesterone, estradiol, prolactin, FSH and LH indicate that capillary blood sampling may be used as an acceptable alternative to venous sampling in the determination of systemic concentrations of these hormones both at rest and following exercise. Bias indicated that capillary concentrations were significantly lower than venous concentrations of total testosterone (1.7 $nmol \cdot l^{-1}$), progesterone (1.49 $nmol.l^{-1}$), and estradiol (0.008 $nmol.l^{-1}$), and significantly higher than venous concentrations for prolactin (-0.016 $nmol.l^{-1}$). However, this systematic bias was relatively small compared to the range of concentrations measured in all cases (Table 1). As such, it may not be necessary to apply a correction factor to account for these differences, particularly as in an applied setting it is the change over time in samples attained via the same method that is the important factor.

The present data suggest that concentrations of total and free IGF-1 in venous blood and capillary blood do not agree. The moderate correlation ($r = 0.472$, $P < 0.01$), large standard deviation and wide LOA between venous and capillary total IGF-1 concentrations indicates a large amount of variability and random error in the relationship between venous and capillary concentrations. A stronger relationship was demonstrated between venous and capillary concentrations of free IGF-1 than total IGF-1 ($r = .611$, $P < 0.01$), although ratio LOA were wider than those of total IGF-1. Overall, venous concentrations of total IGF-1 were lower than capillary concentrations, and venous concentrations were higher than capillary concentrations for free IGF-1, with the differences in concentrations being significant. Lack of agreement for free IGF-1 could be explained by differences in the proportion of IGF-1 bound to one of the six different IGFBPs in the venous and capillary circulation. Free or binary complexed IGFBPs are thought to exit from the circulation rapidly, while ternary complexed IGFBPs seem to be confined to the vascular compartment (Firth & Baxter, 2002), potentially leading to a more variable and changeable environment at the capillaries and lower capillary concentration of free IGF-1 in comparison to venous concentrations. Hence, difference in free IGF-1 concentrations might be expected between venous and capillary blood samples. However, since concentrations of total IGF-1 between sampling methods do not agree either, the role of IGFBPs is likely to be a secondary to other, unidentified, reasons for a lack of agreement.

In preference to capillary blood sampling, saliva sampling has frequently been used to assess hormone concentrations in surrounding exercise. However, establishing the relationship between venous and capillary concentrations is useful because, although saliva provides an easy and completely non-invasive alternative for certain hormones,

there are disadvantages in the use of saliva. For example, the consumption of food or drink high in sugar content or caffeine, as often used in an exercise training environment, can compromise antibody-antigen binding and enzyme activity, while blood contamination of saliva by oral abrasions or microinjuries (e.g. common to contact sports or the wearing of gum shields) may substantially influence results (Papacosta & Nassis, 2011). There are also situations where collecting saliva can be time-consuming and may generate insufficient sample volumes for assay (Granger *et al.,* 2007). In addition, salivary assays are not available for as wide a range of biomarkers as there are for plasma and serum.

It is possible that individual ‘calibration’ coefficients should be developed in order to establish more accurate reflection of venous concentrations for a given individual. However, this would only be important in situations where knowledge of venous concentration is required, whereas in an applied sporting environment, the pattern of change over time may be sufficient in informing training and the physiological status of the athlete. In addition, the reproducibility of the nature of this relationship within an individual remains to be examined.

4.5 Conclusion

In conclusion, the findings of the present investigation indicate strong correlation and agreement between venous and capillary hormone concentrations for cortisol, total testosterone, progesterone, estradiol, prolactin, FSH and LH. Capillary sampling might, therefore, be used as an acceptable alternative to venous blood sampling in determining concentrations of these hormones.

- Chapter 5 -

Agreement between Venous and Saliva Hormone Concentrations at Rest, and in Response to Resistance and Sprint Exercise

5.1 Introduction

Concentrations of circulating testosterone and cortisol are known to increase in response to exercise stimuli (Kraemer et al., 1990). Despite the debate relating to whether such responses to exercise influence training adaptation (Kraemer & Ratamess, 2005; Spiering *et al.,* 2008; West *et al.,* 2010; Wilkinson *et al.,* 2006), these hormones are regularly monitored in elite sport. The traditional reference method for measuring hormone concentrations has been venous blood; but this can be impractical in some applied training situations. Saliva sampling methods are accepted as a more convenient and less invasive technique that enable more frequent measurement of hormone concentrations outside of a laboratory setting.

Validation studies have reported moderate to strong correlations between total venous cortisol and salivary concentrations (r = 0.6 to 0.9) (Lac *et al.,* 1993). Correlation between venous and saliva testosterone concentrations has been shown to be weaker than for cortisol, at r = 0.57, 0.60 and 0.61 for total, free, and bio-available fractions of plasma testosterone respectively (Crewther *et al.,* 2010). Furthermore, few studies have sought to determine whether the correlation between venous and saliva is maintained in response to a physiological stress. This is surprising given that exercise results in a disproportionate increase in blood free and salivary cortisol compared to total cortisol because of saturation of corticosteroid-binding globulin (Paccotti *et al.,* 2005; Vining *et al.,* 1983). Only Vanbruggen *et al.* (2011) have investigated the agreement between

venous and saliva cortisol pre- and post- low, medium and high intensity exercise demonstrating a significant increase in cortisol concentration in both media following high intensity exercise only. There was a significant moderate (r = 0.73) correlation between all matched samples, with saliva concentrations closely tracking those of venous concentrations at lower concentrations, but not as well at higher concentrations. More recently, agreement between serum and salivary testosterone concentrations has been assessed following 30 min cycling at 40%, 60% and 80% VO_2max with strong correlations reported at moderate (r = 0.912) and high (r = 0.898) intensities, while at low intensities an increase in serum but not salivary testosterone was observed (Lane & Hackney, 2014). No research has, as yet, looked at the agreement following performance of high intensity interval exercise.

One of the challenges in studies attempting to determine agreement between concentrations in different fluid samples is that it is often necessary to use different assay methods for the different fluid samples. As a result, measured concentrations might agree in relative terms, but they may not have absolute comparability (Crewther & Cook, 2010). To address this, a relative assessment of samples is required in order to establish whether there is parity between concentrations attained from the two sample methods, such as investigating whether salivary concentrations maintain a consistent relative proportion of venous total concentrations.

In order to establish whether saliva sampling methods can be used as an acceptable alternative to venous sampling in the context of exercise training, the purpose of this investigation was to assess, at rest and following exercise, the agreement between

venous concentrations of total cortisol, total and free testosterone and saliva concentrations of cortisol and testosterone.

5.2 Materials and Methods

5.2.1 Subjects

Eleven physically active men (age 28 ± 3 y; body mass (BM) 77.5 ± 8.3 kg; $\dot{V}O_2$ max 57.2 ± 9.0 ml·kg BM^{-1}·min^{-1}) provided written informed consent before participating in the study, which was approved by the Bath Local National Health Service Research Ethics Committee. Participants had a range of sporting backgrounds, and habitual training varied from light (2-3 sessions per week) to substantial (8-10 sessions per week).

5.2.2 Preliminary Measurements

Preliminary tests were conducted in order to determine each participant's maximal oxygen uptake. Participants completed an incremental test to exhaustion on a SRM cycle ergometer (Schoberer Rad Messtechnik, Fuchsend, Germany). The protocol included a 10 min warm-up at self-selected intensities followed by consecutive 3 min stages, at the end of which the load on the ergometer was increased by 30 W. In the final minute of each stage, a one minute expired air sample was collected and analysed for concentrations of O_2 and CO_2 to assess oxygen uptake. Three maximal repetitions on each resistance exercise of leg press, seated bench press and seated bench pull were then also completed on the concept 2 dynamometer (Concept2 DYNO, Notts, UK). Data were used to calculate work intensities for the subsequent experimental trials. All participants continued their habitual training throughout the study period but were asked to refrain from strenuous exercise and avoid both alcohol and caffeine consumption during the 24 h prior to any main trial.

5.2.3 Experimental Design

Participants completed up to three trials in a randomised order separated by 5 - 10 days and were randomly allocated into two groups. One group completed two trials consisting of two exercise bouts (a cycling sprint interval exercise bout and a resistance exercise bout) separated by 5 h. The sprint interval exercise and the resistance exercise protocols are described in Chapter 4. The second group completed three trials: a rest day; a rest day with carbohydrate-protein supplementation in addition to their normal diet; and a day on which they performed cycling sprint interval exercise. Exercise and protein intake have previously been shown to influence hormone concentrations (Crewther, *et al.,* 2006; Kraemer & Ratamess, 2005; Volek, 2004; Volek, *et al.,* 1997).

5.2.4 Experimental Protocol

Participants arrived in the laboratory at the same time of day for each trial. They continued their habitual training throughout the study period but refrained from strenuous exercise and avoided alcohol and caffeine consumption in the 24 h prior to main trials. Venous blood and saliva samples were obtained on arrival at the laboratory. Participants in the first group completed a bout of exercise (sprint interval exercise or resistance exercise) after baseline sampling, and then performed the other exercise bout (either sprint or resistance) 5 h later. Samples were taken immediately and 1 h after each exercise bout. In the second group, during the rest trial, further samples were obtained at 2, 4 and 6 h. In the protein trial, participants consumed a carbohydrate-protein drink (carbohydrate and whey protein isolate; 1:1 ratio; 0.5 $g{\cdot}kg^{-1}$ protein) immediately after baseline sampling, and further samples were obtained at 1, 2, 4 and 6 h. Participants consumed an additional 0.17 $g{\cdot}kg^{-1}$ protein following sampling at 1, 2 and 4 h to provide a total of 1.0 $g{\cdot}kg^{-1}$ protein supplementary to their diet. In the cycling interval

trial, exercise was performed immediately after baseline sampling, and subsequent samples were obtained immediately post exercise and at 1, 2 and 4 h post-exercise.

5.2.5 Sampling and Analysis

At each sampling time point, venous blood samples were obtained first, followed by capillary sampling, with saliva samples the last to be collected. This order allowed for a lag time between collection of venous and saliva samples of approximately 10 min which has been shown to be sufficient to reflect equilibration between the two sample media (Crewther *et al.,* 2010a; Crewther *et al.,* 2010b). Saliva samples were collected by passive drool into sterile plastic containers (Sarstedt, UK). Participants were asked to refrain from eating or drinking in the 30 min prior to sample collection and rinsed their mouth with water 5 min prior to collection (Shirtcliff *et al*., 2002). Saliva was centrifuged, aliquoted into sterile containers and stored at -20°C until analysis. Venous blood samples were taken from a superficial antecubital vein. Samples were left to clot for 15 min, centrifuged and serum was stored at -20°C until analysis.

Saliva was analysed for testosterone and cortisol concentrations by high-sensitivity enzyme linked immunosorbent assay (ELISA) (Salimetrics Europe, Suffolk, UK). Sensitivity of the assays was: testosterone < 1.0 pg·ml^{-1}, and cortisol < 0.03 μg·dL^{-1}. Intra and inter assay CV for testosterone was 4.6 and 8.5%, and 3.5 and 5.1%, for cortisol.

Serum was analysed for total testosterone, free testosterone, and total cortisol concentrations. Serum total testosterone concentrations were determined via Isotope Dilution Liquid Chromatography Tandem Mass Spectrometry (LC-MS-MS).

Quantitation of results was performed by an internal standard method using Analyst® software version 1.4. Reporting range for the assay was between 0.7 - 60.0 nmol·l^{-1}. Serum concentrations of cortisol were determined by solid-phase, competitive chemiluminescent enzyme immunoassay (Immulite®). Serum free testosterone concentrations were determined by ELISA (ALPCO Diagnostics, Salem, NH, USA). Intra and inter assay CV was 8.9 and 8.8% respectively. Analysis methods for the different media were selected based upon being considered valid and established measurement methods.

5.2.6 Statistical Analysis

Normality tests revealed non-normal distribution of all data. As such, Spearman correlations were used to identify the relation between concentrations from different samples. Bland-Altman analyses were used to determine 95% limits of agreement (LOA) between saliva and capillary samples with their venous sample equivalent. As some degree of heteroscedasticity was evident in all Bland-Altman difference plots, log transformation and subsequent back transformation of the data was used to establish LOA, with limits also expressed as ratio LOA (Atkinson & Nevill, 1998; Bland & Altman, 2007). Paired t-tests were used to determine differences between sampling methods. Following calculation of the bias during Bland-Altman analysis, saliva concentrations were calculated as a percentage of venous concentrations. Saliva concentrations were then scaled according to this percentage to allow for absolute comparison between sampling methods. Data are presented as means and standard deviations with significance accepted at an alpha level of $P \leq 0.05$.

5.3 Results

Hormone concentrations measured following carbohydrate-protein supplementation were not different from those measured during the rest trial, and these data were therefore combined for analysis. A significant increase in total cortisol and testosterone ($P < 0.05$) occurred in response to interval exercise, with a small non-significant increase in total and free testosterone and significant decrease in ($P < 0.05$) cortisol in response to resistance exercise (Fig. 5.1 & 5.2).

Correlation between venous and saliva cortisol concentrations was strong and was similar at rest (r = 0.768, $P < 0.01$) and following exercise (r = 0.804, $P < 0.01$). Bias was significantly greater following exercise ($P < 0.01$) than at rest, however, LOA were similar. Correlation between venous total and saliva testosterone concentrations samples was moderate to strong, being and slightly stronger at rest (r = 0.554, $P < 0.01$) than following exercise (r = 0.434, $P < 0.01$). Bias and LOA were similar for samples obtained at rest and following exercise, with venous concentrations measuring significantly higher than saliva concentrations ($P < 0.01$) (Table 5.1). Correlation between venous free and saliva testosterone concentrations was weak both at rest (r = 0.164, $P > 0.05$) and post-exercise (r = 0.263, $P < 0.05$). Bias and LOA were similar for samples obtained at rest and following exercise (Table 5.1).

Heteroscedasticity was evident in all difference plots (Fig. 5.3) demonstrating a directly proportional increase in the difference between venous and saliva concentration in relation to the increase in the mean value of the two sample concentrations. However, saliva cortisol concentrations were consistently 2.0 ± 0.9% of venous cortisol

concentrations, saliva testosterone concentrations were 2.1 ± 1.0% of venous total testosterone concentrations and saliva testosterone concentrations were 12.9 ± 14.6% of venous free testosterone concentrations. Saliva concentrations were scaled according to these percentages, which substantially reduced the bias and LOA compared with the original concentrations (Table 5.1), with no significant difference between venous concentrations and scaled saliva concentrations for cortisol and total testosterone ($P > 0.05$). However, differences between saliva concentrations and scaled venous concentrations of free testosterone were significant ($P < 0.05$).

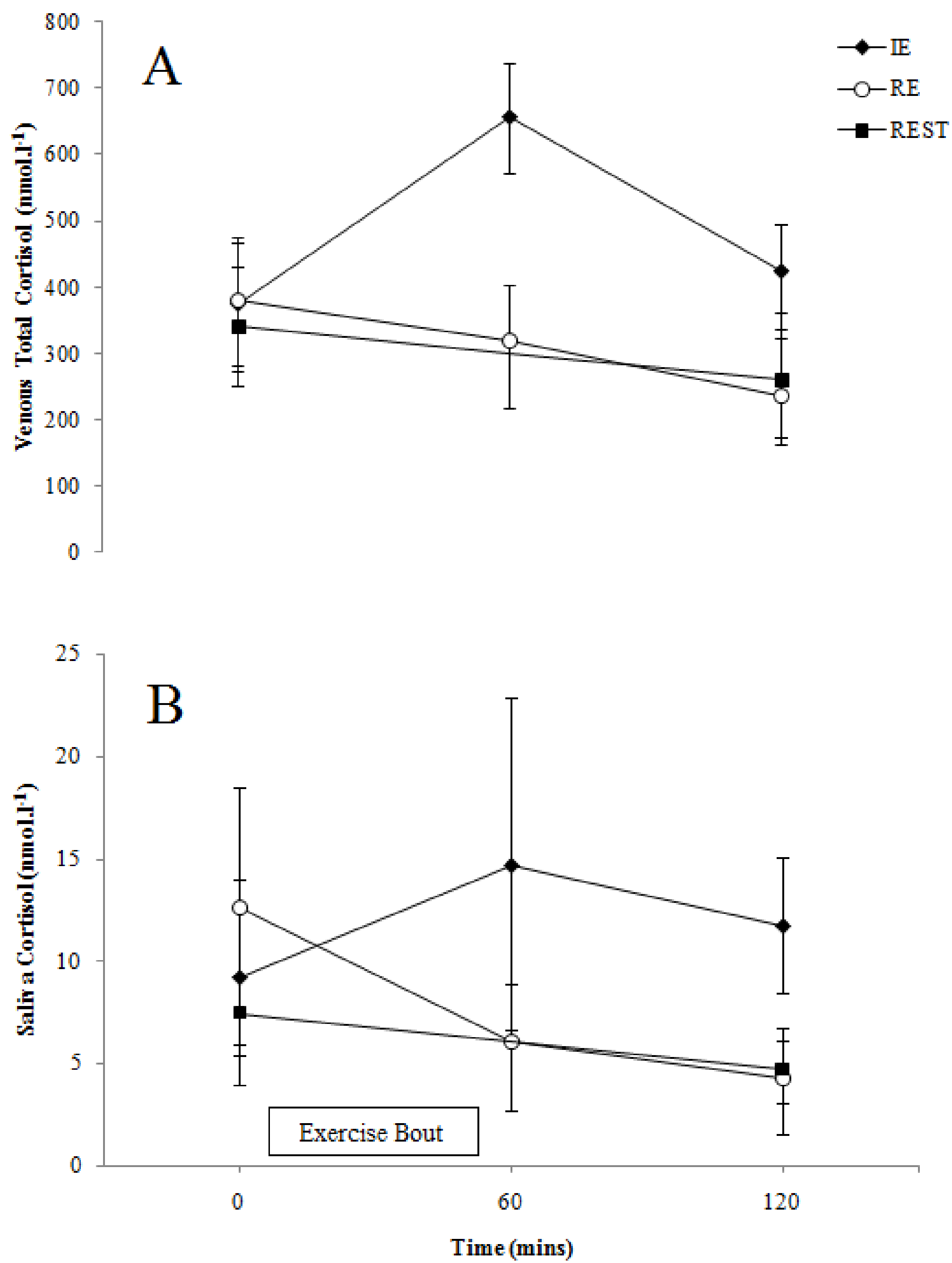

Figure 5.1 Change in cortisol concentration in (A) venous blood (B) saliva with rest, interval exercise (IE) and resistance exercise (RE) at pre-exercise, post-exercise and 1-h post-exercise.

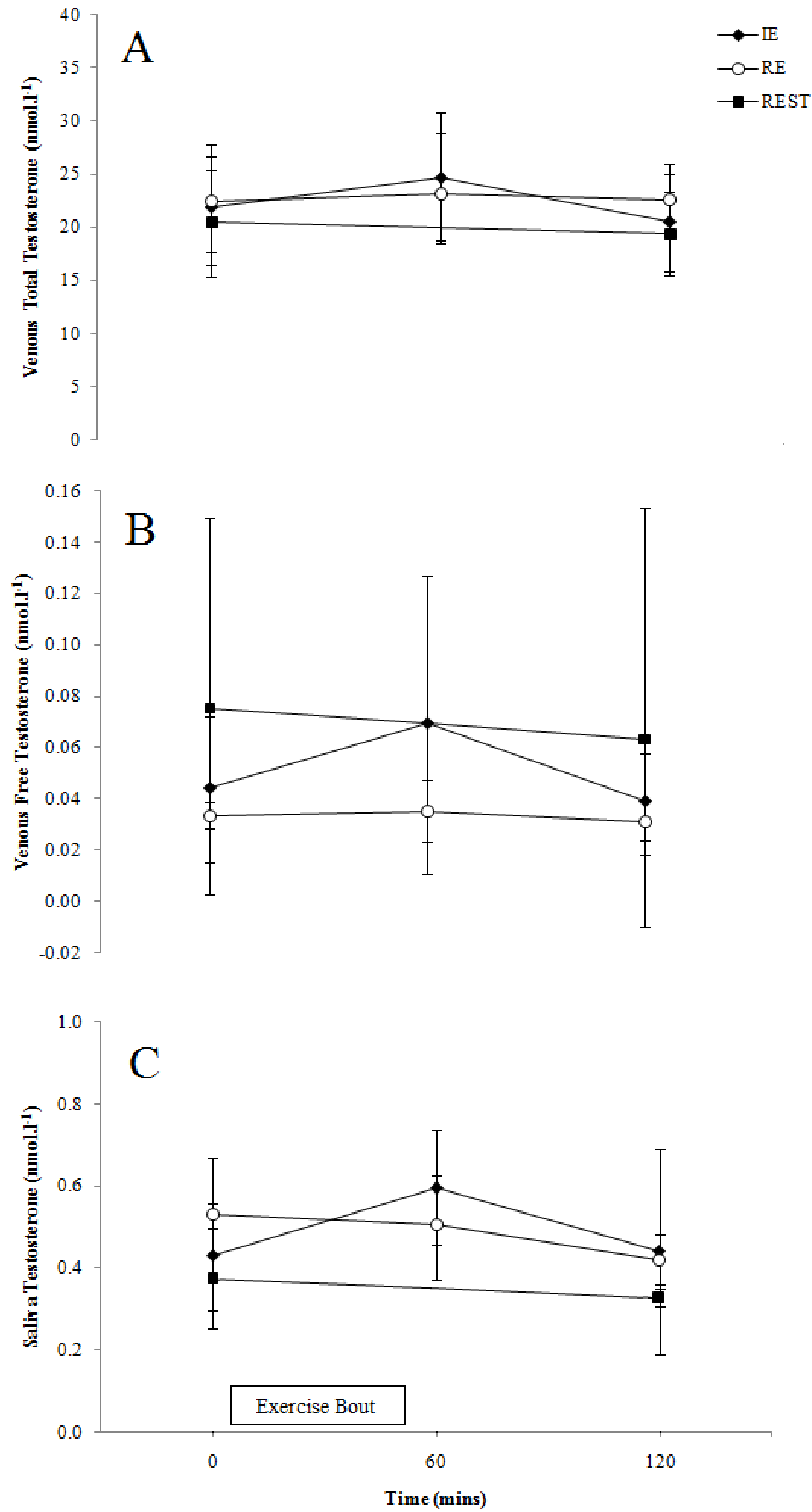

Figure 5.2 Change in (A) total testosterone concentration in venous blood (B) free testosterone concentration in venous blood and (C) saliva testosterone concentration with rest, interval exercise (IE) and resistance exercise (RE) at pre-exercise, post-exercise and 1-h post-exercise.

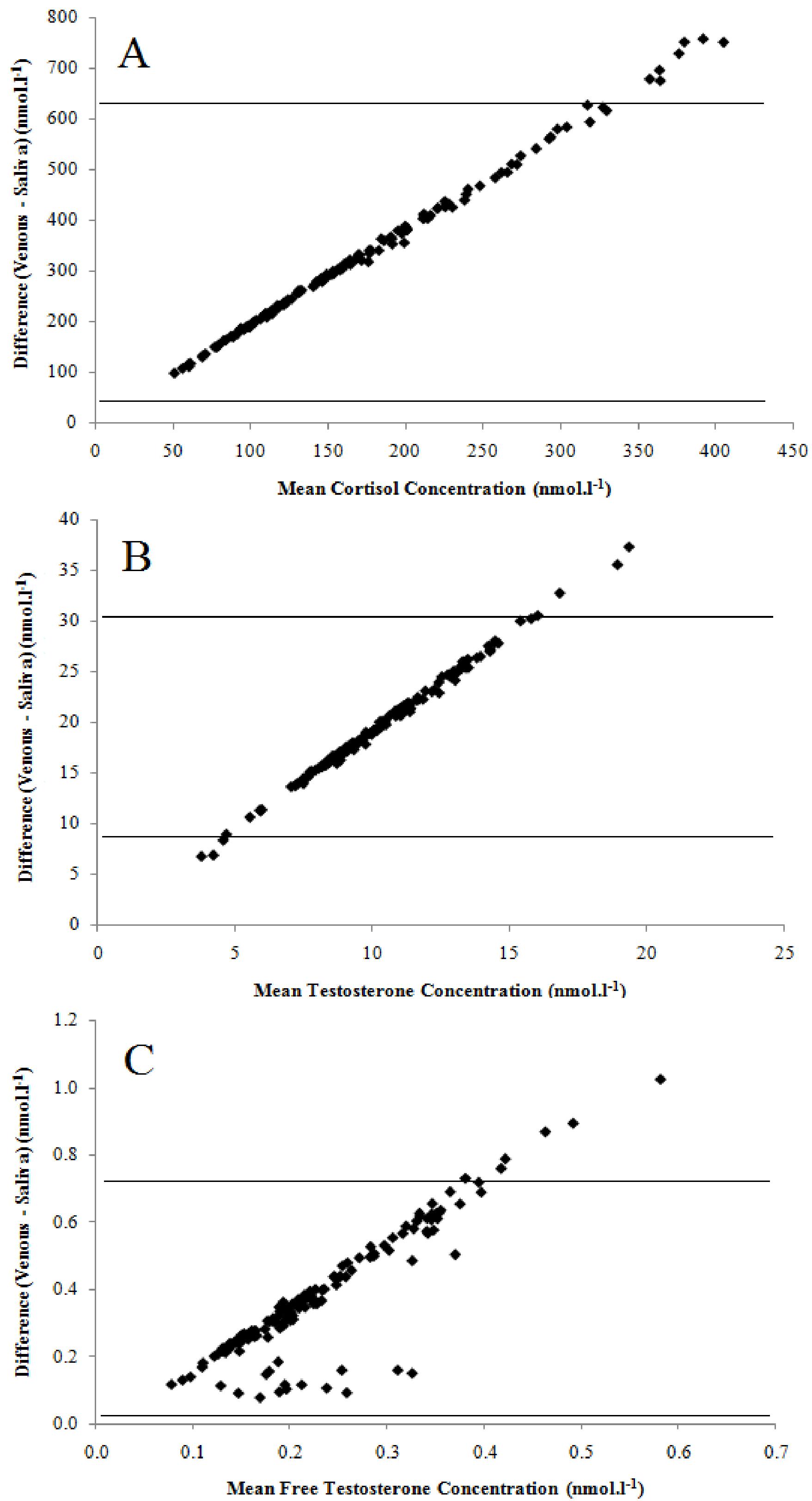

Figure 5.3 Bland-Altman difference plot demonstrating bias and 95% LOA between venous and saliva concentrations of (A) cortisol (B) testosterone and (C) free testosterone concentrations for all samples.

Table 5.1 Correlation and LOA between venous blood samples and saliva samples for all samples, rest, exercise and for males and females. (n = number of samples; Bias = venous concentration – saliva concentration).

Hormone	Condition	n	Range (nmol.l^{-1})	Correlation	Bias (nmol.l^{-1})	LOA (nmol.l^{-1})	Ratio LOA	Scaled Bias (nmol.l^{-1})	Scaled LOA (nmol.l^{-1})	Scaled Ratio LOA
Cortisol	All	144	0.4 - 780.0	.821**	326.1**	29.9 - 622.3	33.9 - 94.9	-23.6	-391.4 - 344.3	0.67 - 1.88
	Rest	80	0.4 - 526.0	.768**	272.3**	79.0 - 465.5	35.3 - 96.7	-17.2	-330.3 - 295.9	0.70 - 1.91
	Exercise	64	0.6 - 780.0	.804**	393.3**†	46.2 - 740.5	32.2 - 92.9	-25.5	-442.1 - 391.2	0.64 - 1.84
Venous Total Testosterone v Saliva Testosterone	All	144	0.1 - 38.0	.528**	19.9**	9.7 - 30.1	35.3 - 73.3	0.6	-13.7 - 14.9	0.74 - 1.54
	Rest	80	0.1 - 33.2	.554**	19.0**	9.8 - 28.2	41.3 - 75.2	0.3	-11.6 - 12.1	0.84 - 1.53
	Exercise	64	0.2 - 38.0	.434**	20.9**	9.8 - 32.0	30.6 - 69.4	1.1	-14.3 - 16.5	0.63 - 1.43
Venous Free Testosterone v Saliva Testosterone	All	144	0.0 - 0.1	.186*	-0.4**	0.0 - 0.7	0.05 - 0.19	0.07*	-0.7 - 0.8	0.69 - 2.83
	Rest	80	0.0 - 0.1	.164	-0.3**	0.0 - 0.6	0.05 - 0.24	0.06	-0.6 - 0.7	0.54 - 2.54
	Exercise	64	0.0 - 0.2	.263*	-0.4**	0.0 - 0.8	0.04 - 0.13	0.06	-0.7 - 0.8	1.01 - 2.98

*$P < 0.05$, **$P < 0.01$, # = Different from all samples, † = Different from rest.

Table 5.2 Saliva concentrations expressed as a percentage of venous serum concentration for total testosterone and cortisol and venous serum free testosterone concentration as a percentage of saliva testosterone concentration for each individual. (Where SD = 0, only one immediate post-exercise time-point for the individual).

	Saliva Cortisol and Venous Total Cortisol (%)		**Saliva Testosterone and Venous Total Testosterone (%)**		**Venous Free Testosterone and Saliva Testosterone (%)**	
Participant	**Rest**	**Exercise**	**Rest**	**Exercise**	**Rest**	**Exercise**
1	3.6 ± 2.2	2.0 ± 1.1	2.1 ± 0.9	2.0 ± 0.6	7.9 ± 4.2	6.5 ± 1.4
2	1.6 ± 0.4	1.8 ± 0.6	1.4 ± 0.2	1.5 ± 0.4	8.6 ± 1.4	7.5 ± 1.2
3	2.2 ± 0.8	2.2 ± 0.0	1.8 ± 0.3	2.1 ± 0.0	11.6 ± 2.1	14.7 ± 0.0
4	1.9 ± 0.9	3.1 ± 1.3	1.8 ± 0.4	2.2 ± 0.5	8.2 ± 1.6	6.2 ± 0.9
5	1.4 ± 0.4	1.6 ± 0.0	1.8 ± 0.2	2.4 ± 0.0	54.6 ± 9.9	59.1 ± 0.0
6	2.2 ± 0.6	2.4 ± 0.2	3.1 ± 0.9	3.5 ± 2.9	5.5 ± 1.6	4.6 ± 1.7
7	2.1 ± 0.8	1.6 ± 0.4	2.5 ± 0.4	2.6 ± 0.7	6.5 ± 0.7	10.5 ± 5.8
8	2.1 ± 0.8	0.8 ± 0.3	2.1 ± 0.3	1.9 ± 0.3	9.5 ± 2.3	12..7 ± 2.8
9	1.6 ± 1.1	1.9 ± 0.9	3.1 ± 0.6	3.1 ± 0.8	3.8 ± 0.7	4.0 ± 1.2

5.4 Discussion

Correlation between venous concentrations of total cortisol, total testosterone, free testosterone and saliva equivalents using the two sampling methods show good, moderate and poor relation respectively. After scaling of saliva concentrations, there was good agreement between venous and saliva compartments for total testosterone and cortisol.

Correlations between venous total and saliva cortisol and testosterone concentrations were strong and moderate respectively. Correlations were not different between rest and following exercise for cortisol, and slightly stronger at rest for testosterone. However, the bias was greater, and absolute LOA for both hormones were wider, following exercise than at rest for both hormones. These differences may be due to calculating the LOA from a smaller number of samples across a wider measurement range following exercise, although it may, alternatively, indicate differences in equilibration of cortisol and testosterone between blood and saliva at rest and following exercise. Differences in the relative increase of venous and saliva cortisol concentrations from pre- to post-exercise (Crewther *et al.,* 2010; Gozansky *et al.,* 2005) have been attributed to saturation of cortisol binding globulin at serum concentrations over 500 $nmol{\cdot}l^{-1}$, leading to a greater relative increase in the free fraction of the hormone. Saliva hormone concentration is considered to reflect the free (unbound) fraction of a hormone (Lac *et al.,* 1993; Laudat *et al.,* 1988; Peake *et al.,* 2005) and therefore saturation of binding globulins might explain a greater relative increase in saliva cortisol concentration. In the present study, the relative increase in saliva cortisol from pre- to immediately post-exercise was greater than that of venous cortisol (75% and 57% respectively), although this was not significant. Subsequent analyses revealed saliva cortisol concentration to be

1.9 ± 0.9% of venous cortisol concentrations at rest and 2.2 ± 0.9% immediately post-exercise suggesting that regardless of absolute serum concentration, approximately the same proportion of cortisol is consistently transferred into saliva.

Saliva testosterone concentrations increased by 33% from pre- to post-exercise, which was significantly greater than the 11% increase observed in venous concentrations. In addition, saliva testosterone concentrations were 6.6 ± 2.1% of venous total testosterone concentrations at rest, but were significantly greater (9.0 ± 5.2%) immediately post-exercise. These findings raise the possibility that saturation of binding globulins or albumin in the blood may occur at high concentrations of testosterone, resulting in an increase in the free fraction of the hormone. However, mean serum free testosterone was 0.05 $nmol \cdot l^{-1}$ at rest and 0.04 $nmol \cdot l^{-1}$ following exercise, indicating that there was not an increase in testosterone in the free form in venous blood. An alternative explanation is that testosterone bound to albumin dissociates readily at the capillaries due to conformational change of albumin upon interaction with the endothelial wall, allowing a greater amount of the hormone to diffuse into the saliva (Pardridge, 1986). This bioavailable testosterone does not contribute to the measurement of free testosterone in the serum, but has been shown to increase, along with an increase in albumin, following exercise (Crewther *et al.,* 2010). It is also possible for a small proportion of albumin itself to enter the oral cavity, possibly transporting testosterone with it (Lac, 2001). These factors are likely to influence testosterone to a greater extent than cortisol, as only up to 7% of cortisol is bound to albumin, in comparison to up to 50% for testosterone (Pardridge, 1986).

Venous free testosterone concentration represented 13 ± 15% of saliva testosterone concentration. Inter-individual variation for venous free testosterone versus saliva testosterone was greater than that for venous total testosterone and total cortisol in comparison with saliva concentrations. For example, for one participant, venous free testosterone concentration was 54 ± 11% of saliva testosterone concentration (Table 2). This may indicate individual variation in the partitioning or transport of hormones between blood and saliva and represents another factor that may confound the relationship between the different compartments. It is possible that individual 'calibration' coefficients should be developed in order to establish more accurate reflection of venous concentrations for a given individual. This may be particularly important where results are used to inform subsequent athletic training and provide information regarding the status of the athlete. However, the reproducibility of the nature of this relationship within an individual remains to be examined.

A limitation of current analytical methods meant that it was necessary to measure concentrations of cortisol, and total and free testosterone using different methods in different sample types. This limits the ability to make absolute comparison between samples but it is still possible to identify whether there is relative agreement between samples using different collection methods and this makes the understanding of agreement arguably more relevant.

While the present data provide encouraging evidence for the use of saliva, it comes with a caveat that although saliva provides an easy and non-invasive alternative, there are disadvantages in the use of saliva. The consumption of food or drink high in sugar content or caffeine, as often used in an exercise training environment, can compromise

antibody-antigen binding and enzyme activity, while blood contamination of saliva by oral abrasions or microinjuries (e.g. common to contact sports or the wearing of gum shields) may substantially influence results (Papacosta & Nassis, 2011). There are also situations where collecting saliva can be time-consuming and may generate insufficient sample volumes for assay (Granger *et al.,* 2007).

5.5 Conclusion

In conclusion, the findings of the present investigation demonstrate that although absolute LOA did not indicate direct agreement between venous and saliva measures, saliva hormone concentrations did display a proportional relationship with venous concentrations. Scaling of salivary concentrations revealed direct agreement between venous and saliva concentrations. This suggests that saliva samples can be used to track and reflect changes in venous hormone concentrations, but that appropriate correction of saliva hormone concentrations is needed if saliva is to be used as an estimate of venous hormone concentrations of total cortisol and total testosterone.

- Chapter 6 -

Measurement of Steroid Hormones in Saliva: Effects of Sample Storage Condition and Duration

6.1 Introduction

Assessment of hormone responses to athletic activity can provide valuable information regarding training stress, adaptation and exercise performance (Groschl, 2008). Measurement of steroid hormones in saliva is often used as an alternative to blood in studies of elite performers (Filaire *et al.*, 2001; Elloumi *et al.*, 2003; Beaven *et al.*, 2008a; Beaven *et al.*, 2008b; Crewther *et al.*, 2008; Crewther *et al.*, 2010; Edwards *et al.*, 2006). Saliva collection procedures are non-invasive, easy to administer, stress-free and enable repeated sampling over the course of minutes or days (Granger *et al.*, 2004). Furthermore, validation studies have demonstrated good agreement between salivary and total serum hormone concentrations(Crewther *et al.*, 2010); especially the free or biologically active fraction of blood hormones (Vining *et al.*, 1983; Crewther *et al.*, 2010; Arregger *et al.*, 2007).

Factors such as storage duration and temperature can influence saliva concentrations of steroid hormones because of degradation by enzymes within the saliva matrix (Chikuma *et al.*, 1991; Kato *et al.*, 1992), while micro-organisms in the oral cavity are potent decomposers of salivary components (Kaufmann *et al.*, 1999; Suzuki *et al.*, 1998). Cortisol has been reported to be stable at 5°C for up to 3 months or at -20°C and -80°C for up to one year, but to decrease by approximately 9% per month in samples stored at room temperature (Garde and Hansen, 2005). In contrast, others have reported a 25% decrease in salivary cortisol after just 3 weeks of storage at 4°C

or at room temperature, and an alarming 30-70% decrease over this period in samples that were not centrifuged prior to storage (Groschl *et al.*, 2001). Testosterone has been reported to be stable at -80°C for up to three years but to decrease by 18% after 6 months at -20°C and increase by 20% and 330% after 1 week and 4 weeks of storage at 4°C, respectively (Granger *et al.*, 2004). Despite these mixed findings, the general consensus communicated in recent review articles is that salivary steroids are stable for up to 1 month at 4°C (i.e. in a regular household refrigerator) and for up to 3 months at -20°C (Wood, 2009; Gatti and De Palo, 2010; Papacosta and Nassis, 2011).

Saliva collection and analysis for the measurement of hormone concentrations is becoming increasingly routine in elite sport. However, this setting places restrictions on how samples can be handled and stored after collection, especially in relation to access to freezers and centrifuges. As such, samples may be exposed to storage for long periods at room or fridge temperature (e.g. across training camps or during transportation) as whole native saliva before delivery to the laboratory. Therefore, the purpose of this study was to assess the effect of storage condition and duration on the concentration of selected steroid hormones typically analysed in elite sport. Specifically, we investigated the influence of storage temperature at 4°C compared to 20°C on salivary cortisol, testosterone and estradiol concentrations after 1, 3, 7 and 14 days; and the longer-term (28 and 84 days) stability of these hormones in whole saliva when stored in freezer conditions (-20°C and -80°C).

6.2 Methods

6.2.1 Participants

Saliva samples were obtained from healthy male (n = 6) and female (n = 3) adult volunteers aged 21-39 years. Ethical approval was granted by the local National Health Service Research Ethics Committee, in line with the Helsinki Declaration of 1975.

6.2.2 Preliminary Investigation

An initial pilot study was used to inform the methods used in the present investigation. Specifically, we aimed to determine the volume of saliva that could be realistically provided by an individual in a single sample as well as the effects of specimen collection duration and multiple aliquoting of the same sample on sample concentration. Participants (n=2) were asked to produce consecutive 6 mL saliva samples into 10 mL containers. Participants were able to provide 18 mL of saliva within 15-30 min. Each sample was spilt into six aliquots of 1-mL. The entire first sample, and half of the second and third samples were vortexed and aliquoted in raw form, while the remainder of the second and third samples were centrifuged prior to being aliquoted. Samples were frozen immediately at -80°C and stored overnight before analysis of testosterone, cortisol, and estradiol by enzyme-linked immunosorbent assay (ELISA; Salimetrics, PA, USA). Variation in concentration between pre-storage treatment and between individual aliquots of the same sample was small, and within 95% confidence intervals based on the intra-assay coefficient of variation (CV). These results showed that measured hormone concentrations were not influenced by sequential aliquoting from a sample or by the vortexing process prior to aliquoting.

6.2.3 Experimental Procedures

To study the short-term effects of sample storage in a regular household refrigerator compared to room temperature (i.e. conditions indicative of those typically available at field-based testing sites in elite athletes) and the longer duration stability of hormones in samples stored in freezer conditions (i.e. -20°C and -80°C), participants provided saliva samples on two separate testing days (separated by 1 week). On each occasion, participants provided two 10 mL saliva samples via unstimulated passive drool into 15 mL collection tubes with no additive. In order to achieve a wide range of hormone concentrations, we took advantage of the circadian variation in hormones by collecting samples from all participants both upon waking and in the late afternoon on each testing day; providing 18 different samples over a range of concentrations for storage in each condition. To minimise any contamination of samples, participants were asked to refrain from brushing their teeth and eating or drinking in the 60 min prior to sample collection. Participants were also required to rinse their mouths with water 10 min prior to sampling.

Following collection, samples were vortexed prior to being separated into eight 1-mL aliquots and stored in the appropriate condition. Samples collected on the first occasion were frozen at -80°C and -20°C and analysed at baseline and after 28 days and 84 days. Samples collected on the second occasion were stored in a refrigerator at 4°C or at a room temperature of 20°C, and were analysed after 1, 3, 7 and 14 days, and compared to a baseline -80°C control. For consistency in the sequence of aliquoting and assay procedures, all -80°C samples were handled prior to -20°C samples, and all 4°C samples were handled prior to those stored at 20°C. On all occasions, morning samples were processed prior to afternoon samples.

Once samples had been stored for the designated amount of time in the assigned condition, all samples (stored at a higher temperature than -80°C) were transferred to -80°C overnight in order that samples stored at room and fridge temperature completed a freeze-thaw cycle to precipitate the mucins in the sample (Granger *et al.*, 2007) and so that all samples could be defrosted from the same condition. Following overnight freezing at -80°C, samples were defrosted at room temperature, centrifuged for 10 min at 5000 rpm in a micro-centrifuge to remove particulate matter before clear sample was removed and pipetted into separate Eppendorf tubes. Samples were analysed in duplicate for concentrations of testosterone, cortisol, and estradiol by ELISA (Salimetrics, PA, USA). The intra-assay co-efficient of variation was less than 9% for all assays. Inter-assay reliability calculated using the same high and low control samples run in quadruplicate on all plates were 5.4 and 8.4% for cortisol, 11.8 and 18.3% for testosterone, and 17.6 and 16.5% for estradiol.

6.2.4 Statistical Analysis

Two, two-way ANOVA for repeated measures (treatment x time) were used to identify overall differences between experimental conditions; one to assess differences between short-term storage conditions (4^{oC} and 20^{oC}), the second between long term-storage conditions (-20^{oC} and -80^{oC}). The Greenhouse–Geisser correction was used for epsilon <0.75 and the Huynh–Feldt correction was adopted for less severe asphericity. Where significant F values were found, the Holm–Bonferroni stepwise correction was applied to determine the location of variance (Atkinson, 2002). All data are expressed as mean ± standard deviation. Statistical significance was set at the 0.05 level. The likelihood that any observed changes in a given sample

were influenced by sample handling variance was calculated based on the inter-assay CV using the formula of Reed *et al*. (2002).

6.3 Results

6.3.1 *Cortisol*

A main effect was observed for time in both long term (*P* < 0.05) and short term storage conditions (P < 0.05). Cortisol concentrations were significantly reduced relative to baseline measurements (mean ± SD; 2.5 ± 2.1 $ng{\cdot}ml^{-1}$, range: 0.5 – 6.9 $ng{\cdot}ml^{-1}$) under both freezer conditions after 28 days (-9 ± 11%; 2.3 ± 1.9 $ng{\cdot}ml^{-1}$, $P \leq 0.01$) and 84 days (-10 ± 13%; 2.3 ± 1.8 $ng{\cdot}ml^{-1}$, $P < 0.01$) of storage. Under non-freezer conditions, cortisol concentrations did not decrease below baseline (mean ± SD; 2.8 ± 1.9 $ng{\cdot}ml^{-1}$, range: 0.5 – 6.3 $ng{\cdot}ml^{-1}$) until the 14-day time-point (-12 ± 16%; 2.6 ± 1.7 $ng{\cdot}ml^{-1}$, $P < 0.05$). There were no differences between the freezer conditions or between the non-freezer conditions in either the magnitude of the time-course of these changes (Fig. 6.1).

6.3.2 *Testosterone*

A main effect was observed for time in the freezer, long term storage conditions (P < 0.05). Relative to baseline measurements (99.2 ± 31.5 $pg{\cdot}ml^{-1}$, Range: 34.8 – 151.1 $pg{\cdot}ml^{-1}$), testosterone concentrations in freezer conditions were maintained at the 24-day time-point, but had increased significantly above baseline by the 84-day time-point (24 ± 16%; 122 ± 37 $pg{\cdot}ml^{-1}$, $P < 0.001$); with no differences between -80°C and -20°C treatments (Figure 2). In the non-freezer storage conditions a main effect for time were detected (P < 0.05), with a linear decrease in testosterone observed from baseline measurements (95.9 ± 41.2 $pg{\cdot}ml^{-1}$, range: 21.2 – 168.0 $pg{\cdot}ml^{-1}$); reaching its lowest concentrations by day 7 (-26 ± 15%; 73.4 ± 36.2 $pg{\cdot}ml^{-1}$, $P < 0.001$) but returning to near basal concentrations by 14 days (99.8 ± 43.3 $pg{\cdot}ml^{-1}$). A main effect for condition was also present ($P < 0.05$) with the decrease in testosterone

concentrations observed in non-freezer conditions across days 1-7 was significantly greater when samples were stored at 20°C compared to 4°C ($P < 0.001$; Fig. 6.2).

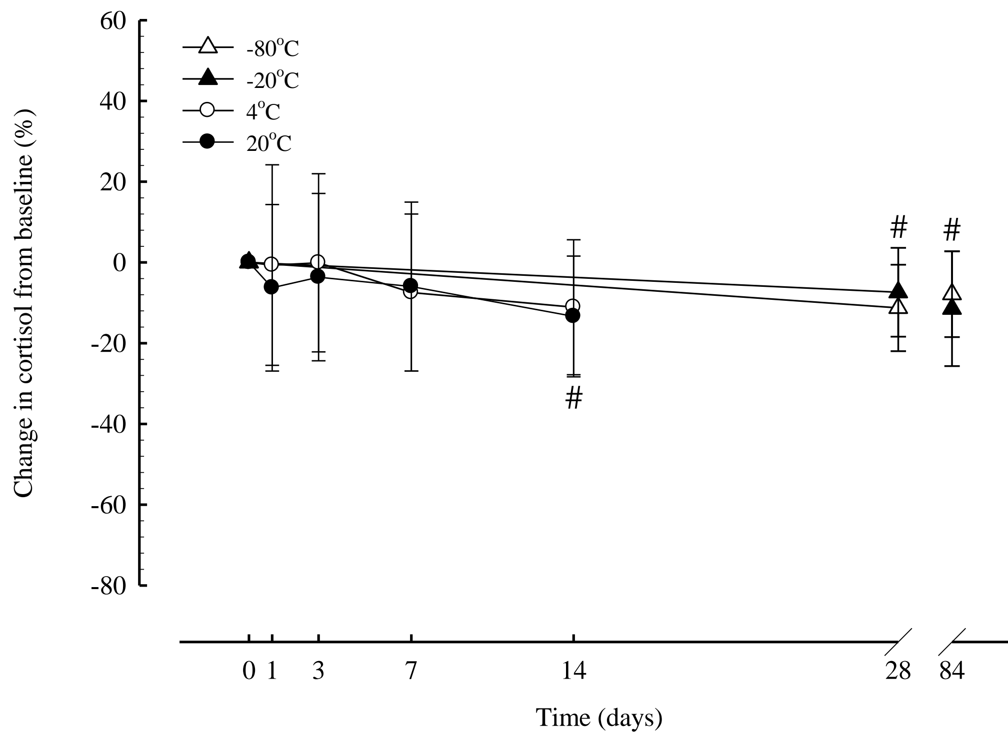

Figure 6.1 Saliva cortisol concentrations expressed as a percentage change from baseline after long-term storage at -80°C and -20°C, and short-term storage at 4°C and 20°C. #, Significantly different from baseline across both treatments.

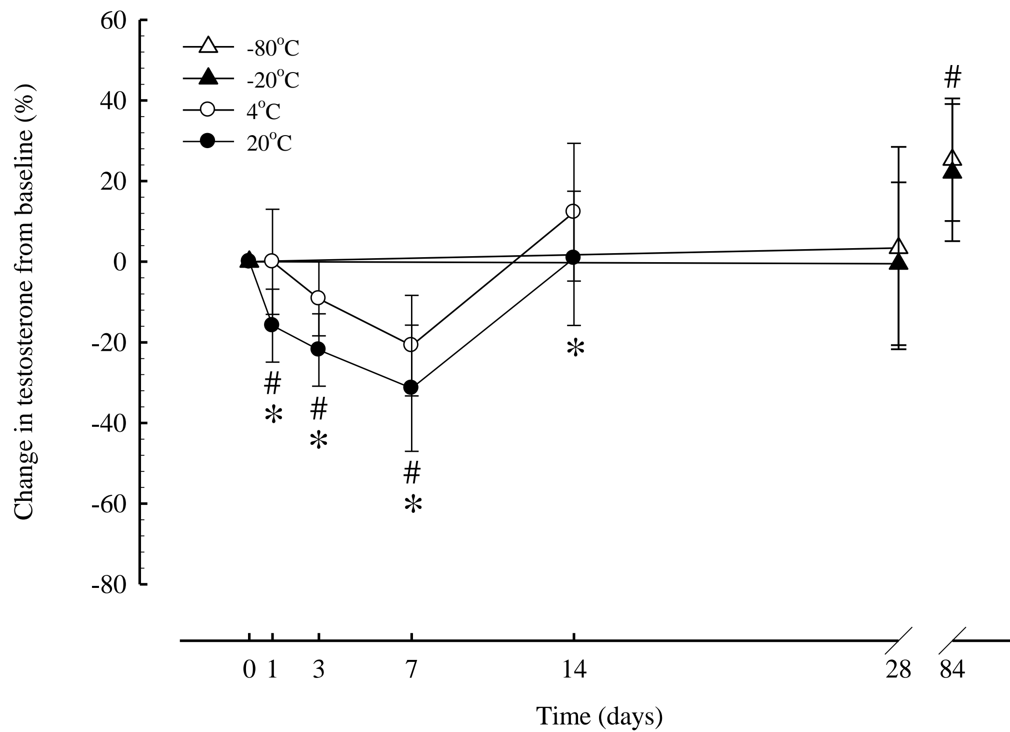

Figure 6.2 Saliva testosterone concentrations expressed as a percentage change from baseline after long-term storage at -80°C and -20°C, and short-term storage at 4°C and 20°C. #, Significantly different from baseline across both treatments. *, Significantly different between treatments.

6.3.3 *Estradiol*

A main effect was observed for time in long term storage conditions ($P < 0.05$) with estradiol concentrations reduced relative to baseline measurements (mean ± SD; 5.0 ± 1.6 pg·ml^{-1}, range: 2.4 – 7.9 pg·ml^{-1}) under both freezer conditions after 28 days of storage (-9 ± 12%; 4.4 ± 1.4 pg·ml^{-1}, $P < 0.01$) but with no differences between -80°C and -20°C treatments at any time-point. A main effect for time was also observed in short-term storage conditions ($P < 0.05$) with estradiol concentrations in both non-freezer conditions reduced relative to baseline concentrations (5.3 ± 1.8 pg·ml^{-1}, range: 2.6 – 9.4 pg·ml^{-1}) by day 1 (-15 ± 21%; 4.5 ± 2.0 pg·ml^{-1}, $P \leq 0.01$), and were substantially decreased below baseline by day 7 (-58 ± 17%; 2.2 ± 1.0 pg·ml^{-1}, $P < 0.001$). Following 14 days storage, estradiol concentrations were greater than reported at day 7, but remained lower than baseline concentrations (-27 ± 35%; 3.6 ± 1.5 pg·ml^{-1}, $P < 0.05$). A main effect for storage condition was also detected in short-term storage conditions ($P < 0.05$) with estradiol concentrations were significantly lower in samples stored at 20°C in comparison to 4°C when measured at day 1 and day 3 ($P \leq 0.01$; Fig. 6.3).

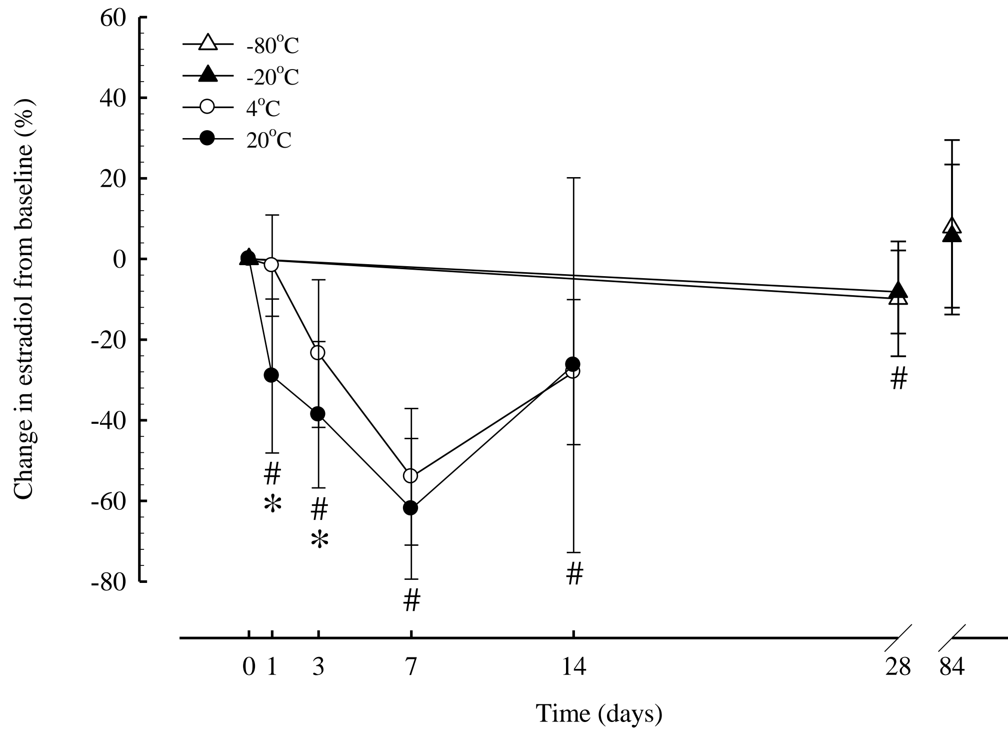

Figure 6.3 Saliva estradiol concentrations expressed as a percentage change from baseline after long-term storage at -80°C and -20°C, and short-term storage at 4°C and 20°C. #, Significantly different from baseline across both treatments. *, Significantly different between treatments.

6.3.4 Inter-Assay Variability

The likelihood that observed changes in hormone concentration were influenced by sample handling variance was calculated based on the inter-assay CV using the formulae of Reed *et al*. (2002). Previous formulae (Wood & Durham, 1980; Wood, 1981) purport a two-fold difference to be the upper limit on acceptable variability. The formula of Reed *et al.* (2002) allows determination of the probability that an assay will accurately discern a k-fold difference between sample analyte concentrations where a sample has repeated measures, based on the knowledge of assay variability (CV). Analysis using the formula indicates that there was a 34% and 36% probability that the decrease in cortisol concentrations observed after 1 and 3 months storage, respectively, in freezer conditions (i.e. a 9.5% mean decrease) was due to inter-assay variation. For testosterone and estradiol samples stored in freezer conditions, an even larger proportion (94% and 72%) of the reported changes in sample concentrations at 1 month was likely to be representative of inter-assay variation, respectively. For samples stored in non-freezer conditions, there was a 24% chance that the decrease in cortisol concentrations at day 14 (-12 ± 16%) was a function of inter-assay variance, while there was a 27% and a 6% probability that the decrease in testosterone (-26 ± 15%) and estradiol (-58 ± 17%) concentrations at day 7 may be ascribed to inter-assay variance, respectively.

6.4 Discussion

The main focus of the present study was to assess the effects of storage temperature and duration on measured concentrations of steroid hormones in whole, native saliva. Cortisol concentrations were not different between aliquots frozen immediately and those stored at room temperature or in a refrigerator for up to 7 days. In contrast, there was a linear decrease in testosterone and estradiol concentrations across 1 to 7 days of storage in non-freezer conditions; but samples stored in a refrigerator were more stable than those stored at room temperature. Longer-term storage of samples in freezer conditions were typically within 12% of baseline levels for all hormones, and a large proportion of these changes may be attributable to inter-assay variance. Storage condition and duration of saliva samples can introduce large error into measured concentrations of salivary hormones; particularly gonadal steroids.

In the present study, there were no effects on the concentrations of salivary cortisol after storage of samples for up to 7 days in a refrigerator or at room temperature. This finding is consistent with reports that cortisol is stable for up to 1 week at 4°C and at 18°C in whole native saliva (Groschl *et al.*, 2001). In contrast, the data reported here shows a linear decrease in testosterone and estradiol concentrations across this period, reaching a mean low of -26 ± 15% and -58 ± 17% at 7 days, respectively. This degradation in sample concentrations was shown to be significantly more pronounced in samples stored at 20°C than at 4°C, for testosterone (at all time-points) and estradiol (at days 1 and 3). These observations are in agreement with previous research showing no differences in cortisol levels after 4 days storage in non-freezer conditions, but a marked reduction in testosterone and estradiol concentrations after 4 days in the refrigerator (18% and 34%) or at room temperature (32% and 64%),

respectively (Schwartz *et al.*, 2005). One plausible explanation is the influence of bacteria on sample integrity, given previous evidence of a decrease in testosterone but not cortisol in samples loaded with bacteria and when stored at room temperature prior to analysis (Whembolua *et al.*, 2006). Thus, our data support the notion that bacterial multiplication may contribute to the decrease in gonadal steroid hormone concentrations during storage at higher temperatures.

Following the marked decrease in measured testosterone and estradiol levels after 1 weeks storage in non-freezer conditions, the subsequent increase or 'rebound' in the concentrations of these hormones reported after 2 weeks was unexpected and, to our knowledge, this pattern has not been reported previously. Other authors have observed an increase in testosterone concentrations in samples stored at 4°C, but this was a linear increase from initial concentrations, and was determined using a single pooled sample from all participants (Granger *et al.*, 2004). The increase in concentrations from day 7 to day 14 in the present study was systematic, with nearly all individual samples demonstrating an increase in concentrations. This effect cannot be explained by assay variation, as control values did not demonstrate the same pattern. It is beyond the scope of this study to determine the reason for this effect, but possible explanations include changes in sample pH, enzymatic activity and bacterial multiplication. Where the pH of the sample drops below 4, the antibody–antigen reaction necessary for accurate measurement of salivary biomarkers by immunoassay is compromised, resulting in artificially high estimates of hormone concentrations (Granger *et al.*, 2007). In urine samples, microbial contamination can induce modification of steroid structure by oxidoreductive reactions (de la Torre *et al.*, 2001). As such, while an increase in steroid concentrations due to bacterial growth is less

frequently observed than a decrease, an increase can occur due to bacterial hydrolysis of androstenediol, followed by 3-beta hydroxysteroid dehydrogenase and steroid isomerase activity (Mareck *et al.*, 2008). This may also apply to estradiol given that conversion from androstenedione also occurs via the action of 3-beta hydroxysteroid dehydrogenase.

It is noteworthy that the long-term storage of steroid hormones in saliva was more stable in samples stored in freezer compared to non-freezer conditions, and that there were no significant differences between a regular house-hold freezer (-20°C) compared to storage at ultra-low temperatures (-80°C). Variation in cortisol concentrations was less than 10% for longer-term storage of samples for up to 3 months, but was shown to be significantly different from baseline. This is in contrast to the findings of others who have shown that salivary cortisol is stable for up to 1 year when archived at -20°C or -80°C (Garde and Hansen, 2005) in centrifuged saliva. However, cell and cell fragments within the oral mucosa contained in whole saliva provide an optimal surface for bacterial growth (Macpherson and Dawes, 1999), so centrifugation prior to storage likely preserves the concentration of hormones within the sample (Groschl *et al.*, 2001; Granger *et al.*, 2004). The present investigation aimed to preserve ecological validity by storing whole saliva as would be done in an exercise training environment; thereby providing greater real-world relevance.

To the authors' knowledge, this is the first study to report on the longer-term stability of salivary estradiol in freezer conditions, and our data show small albeit significant decreases in estradiol concentrations (i.e. less than 10% from baseline) after 1 month, followed by a return to near baseline values by 3 months. Although testosterone

concentrations were shown to be stable after 1 month in freezer conditions, a similar but more marked subsequent increase in concentrations to that observed for estradiol was shown at 3 months. This increase in gonadal steroid concentrations for samples stored in freezer conditions is in contrast to others who have shown an 18% decrease or no-change in measured testosterone levels when stored for 6 months at -20°C or -80°C, respectively (Granger *et al.*, 2004); but may be a function of differences between studies in the duration of sample storage prior to analysis.

It is important to highlight that potential sources of pre-analytical measurement error (such as drift in the quality and concentration of reagents and controls as well as subtle differences in assay technique) may account for some of the variance in hormone levels across time in the present study. As an expression of plate-to-plate consistency, the inter-assay co-efficient of variation calculated from the mean values for the high and low controls on each plate was less than 20% for all analyses; which is considered to be acceptable for immunoassays (Kivlighan *et al.*, 2004; Reed *et al.*, 2002). Application of the formula of Reed *et al*. (2002), as a method to further explore the probability that any observed changes in a given single sample were influenced by inter-assay variation suggests that changes in hormone concentrations across the period of storage needed to be fairly large to be attributable to storage condition *per se*. For example, there was approximately an 80% probability that changes in gonadal steroids after 1 month storage in freezer conditions was due to inter-assay variation. In contrast, the likelihood that the relatively large decrease in testosterone or estradiol concentrations by 7 days of storage at room or fridge temperature was due to sample handling variance was 26% and 6%, respectively; thereby more likely representing a true effect of storage temperature as a source of measurement error.

6.5 Conclusion

The present results on the reliability of salivary steroid hormones under different storage conditions highlights problems that may occur when saliva is not frozen in the hours immediately after collection. As long as these changes remain within the precision of the assay, however, even significant differences are not practically meaningful. Nonetheless, greater changes in hormone concentrations associated with storage condition could be misinterpreted and may, for example, confuse the interpretation of the stress response to training in elite sport. The present findings suggest that, in order to preserve the 'original' concentrations of hormones in saliva, gonadal steroids should be kept refrigerated upon collection and stored at -20°C or below within 24 h. Samples to be analysed for cortisol may be stored or transported for up to 7 days at room temperature or in a refrigerator before freezing. Samples to be analysed for cortisol, testosterone or estradiol may remain frozen for up to 1 month prior to analysis.

- Chapter 7 -

The Influence of Exercise Bout Order on the Concentration of Serum Hormones

7.1 Introduction

The acute hormone response to exercise has been linked to subsequent adaptation to training (Kraemer and Ratamess, 2005; Ahtiainen *et al.*, 2005; Crewther *et al.*, 2006; Beaven *et al.*, 2008a; Beaven *et al.*, 2008b). Exercise elicits a response in a wide variety of hormones with the resultant changes, and interaction between them, influencing processes such as muscle protein turnover and substrate metabolism, as well as being able to identify changes in the physiological status of an athlete through being indicative of non-functional over-reaching and over-training. Testosterone and cortisol are the primary hormones that have been linked to influencing muscle protein turnover with the magnitude of testosterone response to strength training having been suggested to influence gains in muscular strength (Beaven *et al.*, 2008a; Beaven *et al.*, 2008b), with cortisol having been shown to increase protein degradation in muscle cells, suggesting a prominent role in tissue remodelling (Kraemer and Ratamess, 2005). In addition, follicle stimulating hormone (FSH) and luteinising hormone (LH) can act as indicators of the hypothalamus-pituitary-gonadal axis, and LH, as an upstream signal for secretion of testosterone, may influence the ability of testosterone to respond to exercise and contribute to adaptation (Safarinejad *et al.,* 2009). Cell proliferation and tissue growth are also influenced by IGF-1, which is considered to be a potent growth factor (Frystyk *et al.*, 2010). IGF-1 has also been identified to be a useful marker of metabolic stress (Nindl *et al.*, 2007). In its role in this capacity, interactions have also been reported with estrogen and progesterone, with estrogen

and progesterone having been demonstrated to be important in the regulation of substrate utilisation during exercise (Braun & Horton, 2001), as well as exerting an influence on circulating IGF-1 concentrations (Juul *et al.*, 1997; Gleeson & Shalet, 2005). Estrogen has also been shown to influence concentrations of cortisol and prolactin (Kraemer *et al.*, 2012). Prolactin has been suggested to have a role in identification of over-training in athletes, with elevated resting concentrations thought to be indicative of non-functional over-reaching and over-training syndrome, with suppressed prolactin response to a second exercise bout a possible diagnostic tool for over-training syndrome (Meeusen *et al.*, 2010).

In competitive sport it is common practice for athletes to complete at least two training sessions within a day. Depending on the event and requirements of the sport, these training sessions may target very different training goals. An interference theory has been suggested (Docherty and Sporer, 2000) relating to the observed compromised strength gains when strength and endurance are trained concurrently (Bell *et al.*, 2000; Hakkinen *et al.*, 2003; Chtara *et al.*, 2008). Given the reported relationship between acute hormone response to exercise and subsequent training adaptation (Kraemer and Ratamess, 2005; Ahtiainen *et al.*, 2005; Crewther *et al.*, 2006; Beaven *et al.*, 2008b; Beaven *et al.*, 2008a) the acute hormone response in relation to individual exercise bouts may contribute to this cumulative effect. As such, it is important to understand hormonal responses to individual exercise bouts of different types, and how the order of these sessions may influence the hormone responses generated by subsequent sessions.

In contrast to a number of studies detailing responses to various types of single exercise bouts (Kraemer and Ratamess, 2005; Crewther *et al.*, 2006), literature concerning the influence of repeated bouts of exercise is sparse, with even less information available on repeated bouts of exercise of different types. It is not known whether performing two different exercise sessions within a day, and in a different order, may influence the hormone response to these sessions and hormone concentrations across the course of the day. Therefore, the purpose of this study was to assess hormone responses to the completion of interval and resistance exercise within the same day, where one session was performed in the morning, and one in the afternoon.

7.2 Method

7.2.1 Participants

Seven males (age 28 ± 3 y; body mass (BM) 77.6 ± 9.5 kg; $\dot{V}O_2$ max 59.5 ± 8.9 ml·kg $BM^{-1}·min^{-1}$) participated in this study. All individuals were healthy, participating regularly in exercise or substantial training. Each participant was briefed regarding the nature of the study and provided written informed consent prior to commencing any testing. This study was approved by the Bath Local National Health Service Ethics Committee.

7.2.2 Preliminary Measurements

Preliminary tests were conducted in order to determine each participant's maximal oxygen uptake. Participants completed an incremental test to exhaustion on a SRM cycle ergometer (Schoberer Rad Messtechnik, Fuchsend, Germany). The protocol included a 10 min warm-up at self-selected intensities followed by consecutive 3 min stages, at the end of which the load on the ergometer was increased by 30 W. In the final minute of each stage, a one minute expired air sample was collected and analysed for concentrations of O_2 and CO_2 to assess oxygen uptake. Three maximal repetitions on each resistance exercise of leg press, seated bench press and seated bench pull were then also completed on the Concept II dynamometer (Concept II DYNO, Notts, UK). Data were used to calculate work intensities for the subsequent experimental trials. All participants continued their habitual training throughout the study period but were asked to refrain from strenuous exercise and avoid both alcohol and caffeine consumption during the 24 h prior to any main trial.

7.2.3 *Experimental Design*

Participants completed two main trials in a randomised order separated by 5-10 days. Over the 24 h preceding the first of these trials, each participant recorded their usual diet and activity and were subsequently asked to adhere to the same dietary intake and activity pattern in the 24 h prior to their second trial. Participants completed two exercise sessions separated by 4 h. One exercise session consisted of high-intensity interval exercise performed on an SRM ergometer and comprised a 10 min warm up followed by 10 x 30 s sprints with 90 s recovery between each sprint. Participants were asked to aim for a peak power of 150 % power at $\dot{V}O_{2\,max}$ and then try to sustain a power output as close to that as they could for the remainder of each 30-s sprint. The resistance exercise session consisted of 5 sets of 10 repetitions at 80% of maximum on seated bench press, leg press and seated bench pull exercises. Exercises were performed as a superset (no rest interval between exercises) in the aforementioned order, with one minute recovery after each superset. In one trial, participants performed the interval exercise session in the morning and the resistance session in the afternoon, and in the other trial they performed the sessions in the reverse order.

7.2.4 *Experimental Protocol*

Each participant arrived in the laboratory at the same time of day for each trial, not fasted, and a pre-exercise blood sample was obtained from an antecubital forearm vein. Participants then completed the first exercise session and blood samples were taken immediately after and 1 h after completion of this first exercise bout. Six hours after the start of the first exercise session, a further blood sample was taken before participants performed the second exercise session, followed by samples taken immediately and 1 h after completion of the second exercise bout (Fig. 1). In all trials,

participants were permitted to leave the laboratory between exercise bouts to continue with their normal daily routine.

Figure 7.1 Schematic of timescales of both trials. Vertical arrows indicate blood sampling time-points.

Blood was analysed immediately for lactate and glucose using an automated lactate and glucose analyser (YSI 2300 STAT Plus). The remaining sample was divided in to two 5-ml aliquots for plasma and serum. The serum sample was left to clot for 15 min at room temperature before being centrifuged at 3000 rpm (1500 g) for 10 min. Plasma samples were centrifuged immediately at 3000 rpm (1500 g) for 10 min. Serum and plasma were then transferred to labelled eppendorfs and frozen at -20°C until further analysis.

Serum samples were analysed for total testosterone, free testosterone, and total cortisol. Plasma samples were analysed for free and total IGF-1. Serum total testosterone concentrations were determined via Isotope Dilution Liquid Chromatography Tandem Mass Spectrometry (LC-MS-MS). Samples were assayed on an automated LC-MS-MS system controlled by Analyst® software. Quantitation of results was performed by an internal standard method using Analyst® software version 1.4. Reporting range for the assay was between 0.7 - 60.0 $nmol.l^{-1}$.

Determination of serum free testosterone concentrations was completed via ELISA (ALPCO Diagnostics, Salem, NH, USA). Intra- and inter-assay CV was 8.9 and 8.8% respectively Serum concentrations of total cortisol were determined via solid-phase, competitive chemiluminescent enzyme immunoassay using the automated Immulite® system using commercially available kits. Plasma total IGF-1 concentrations were determined using a commercially available kit (Active IGF-I ELISA, DSL-10-5600), involving a simple acid-ethanol extraction procedure in which IGF-I was separated from its binding proteins, with an assay sensitivity of 0.004 $nmol.l^{-1}$. Determination of plasma free-IGF-I concentrations was carried out using a commercially available kit (Active free IGF-I ELISA, DSL-10-9400), with an assay sensitivity of 0.002 $nmol.l^{-1}$ (DSL Europe, Oxford, UK). Intra- and inter-assay CV for total and free IGF-1 were 6.0 and 6.7%, and 3.1 and 9.1% respectively. Serum concentrations of estradiol, progesterone, prolactin, FSH and LH were determined via a semi-automated biochip immunoassay analyser (Evidence Investgator, Randox Laboratories, Co. Antrim, UK). Intra assay CV of the fertility array was 8.5, 10.3, 8.0, 6.3, and 5.7% for estradiol, progesterone, prolactin, FSH and LH respectively.

7.2.5 Statistical Analyses

One-way ANOVA was used to identify differences in hormone concentrations across each trial. Two-way, repeated measures ANOVA was performed to identify differences between trials. Subsequent paired two-tailed t-tests were used to identify the location of any variance. Incremental area under curve (IAUC) was calculated from pre-exercise to 1 h post exercise for each training session and each trial day, and total area under curve (TAUC) using the trapezoid method was calculated for all samples on each trial day. Statistical analyses were performed using IBM SPSS

Statistics (version 20.0.0; IBM, New York, NY) and Microsoft Office Excel for Windows version 2003 software (Redmond, WA, USA). All data are presented as mean ± SD. Statistical significance was accepted at $P \leq 0.05$. Post hoc power calculations were performed where near significant results were observed using G*Power software version 3.1.9.2 (Dusseldorf, Germany).

7.3 Results

7.3.1 Morning Interval Exercise – Afternoon Resistance Exercise

In response to interval exercise in the morning, concentrations of cortisol, total testosterone and progesterone were significantly increased above morning pre-exercise values (Table 7.3, Fig. 7.2-3, 7.9). At 1 h post-exercise concentrations of total cortisol and total testosterone had decreased significantly from immediate post-exercise to pre-exercise values, while prolactin concentration had decreased significantly from immediately post-exercise to below pre-exercise values (Fig. 7.8). Concentration of free testosterone displayed the same trend as cortisol and total testosterone, but this was not significant (P = 0.09 and 0.07 respectively). Total testosterone - cortisol (TC) ratio decreased significantly from baseline immediately post-exercise to pre-exercise values at the 1-h post-exercise time-point (Fig. 7.7). No significant changes were identified in the other hormones analysed.

Following completion of interval exercise in the morning, afternoon pre-exercise concentration of cortisol and free testosterone was significantly lower than morning pre-exercise concentration ($P < 0.05$). When preceded by interval exercise, resistance exercise in the afternoon generated a significant increase in cortisol and free testosterone concentrations from pre-exercise. There was a trend for estradiol concentration to increase, but this did not reach significance (P = 0.06). At 1 h post-exercise cortisol and free IGF-1 concentrations were significantly lower than immediate post-exercise concentration (Fig. 2 & 6). TC ratio was significantly higher than pre-exercise and immediate post-exercise levels (Fig. 7.7).

7.3.2 Morning Resistance Exercise – Afternoon Interval Exercise

In response to resistance exercise in the morning, immediate post-exercise cortisol and prolactin concentrations were significantly lower than pre-exercise, while the TC ratio was significantly higher (Fig. 7.2, 7.7 & 7.8). At 1 h post-exercise, prolactin was lower than pre-exercise values and cortisol was lower than both pre-exercise and immediately post-exercise. The TC ratio was higher than pre-exercise and immediate post-exercise values.

Following completion of resistance exercise in the morning, afternoon pre-exercise concentrations were significantly lower than morning pre-exercise concentrations for total testosterone, free testosterone and total IGF-1 ($P < 0.05$). Afternoon interval exercise generated significant increases from afternoon pre-exercise values in cortisol, prolactin, progesterone and estradiol, while the TC ratio decreased significantly from pre-exercise values. The immediate post-exercise increase in free testosterone from pre-exercise following afternoon interval exercise was not significant ($P = 0.09$). At 1 h post-afternoon interval exercise, cortisol, progesterone and estradiol had decreased significantly ($P = 0.00$) and were not different from pre-exercise values. Prolactin decreased significantly from immediate post-exercise levels, but remained significantly elevated above baseline, The TC ratio remained significantly lower than pre-exercise values.

7.3.3 Influence of Prior Resistance Exercise on Interval Exercise Response

Following completion of resistance exercise in the morning, the magnitude of the response to interval exercise was greater for prolactin (141% *vs* 60%) TC ratio (-42% *vs* -30%), and approached significance for total IGF-1 (9% *vs* 2%; $P = 0.09$) than

when interval training was performed in the morning. IAUC data from pre-exercise to 1 h post-exercise indicated a significantly greater IAUC for prolactin when interval exercise was performed in the afternoon preceded by resistance exercise in the morning than when performed in the morning ($P < 0.01$). The same trend approached significance for FSH ($P = 0.09$).

7.3.4 Influence of Prior Interval Exercise on Resistance Exercise Response

Following completion of interval exercise in the morning, the magnitude of the response to resistance exercise in the afternoon was significantly greater for cortisol (25% *vs* -16%) and prolactin (10% *vs* -26%), and approached significance for TC ratio (-10% *vs* 23%; $P = 0.08$). IAUC from pre-exercise to 1 h post exercise resistance exercise was significantly greater for cortisol (P = 0.02) in the afternoon when preceded by interval exercise than when performed in the morning. It was not different for any other hormone, but there was a non-significant trend for a lesser and greater IAUC for resistance exercise in the afternoon when following interval exercise for TC ratio ($P = 0.08$) and free IGF-1 ($P = 0.09$) respectively.

Total AUC, calculated across the whole trial day from pre-exercise in the morning to 1 h post-exercise in the afternoon, was significantly greater over the morning interval training – afternoon resistance training trial for total testosterone ($P < 0.01$), total IGF-1 ($P = 0.02$), and approached significance for FSH ($P = 0.09$). Total AUC for estradiol was significantly greater over the morning resistance training - afternoon interval training session ($P = 0.04$).

Total IAUC, calculated across the whole trial day from morning pre-exercise concentrations to 1h post-exercise in the afternoon was significantly greater across the morning resistance exercise - afternoon interval exercise trial for cortisol ($P = 0.04$) and prolactin ($P < 0.01$), and significantly greater across the morning interval exercise - afternoon resistance exercise trial for TC ratio ($P = 0.02$), with IAUC across the day for free testosterone approaching significance for this trial ($P = 0.09$).

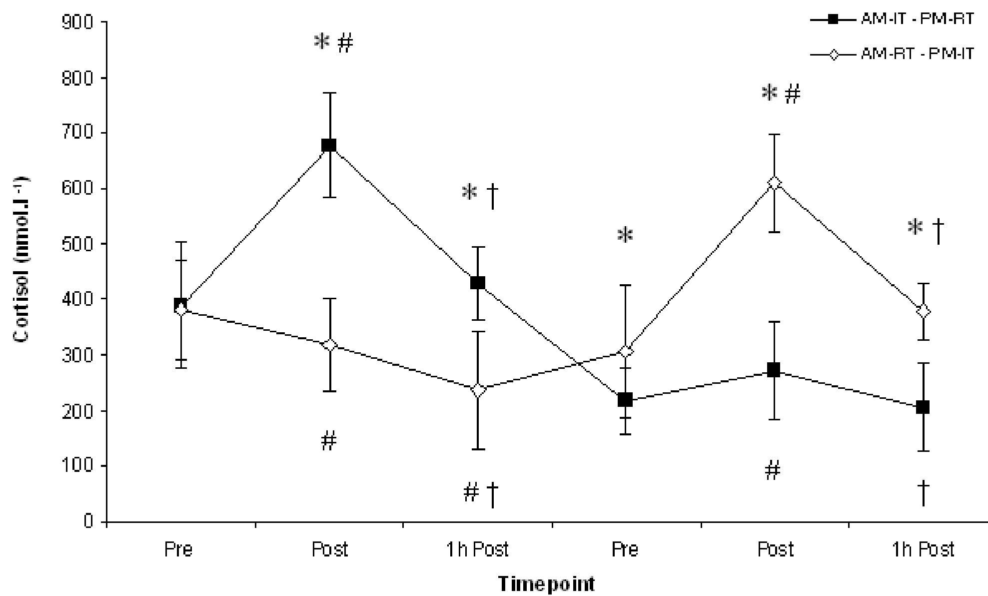

Figure 7.2 Change in cortisol concentration across trials with two exercise bouts (AM-IT – PM-RT = Morning interval exercise, afternoon resistance exercise. AM-RT – PM-IT = Morning resistance exercise, afternoon interval exercise). (* = Difference between trials; # = Difference from pre-exercise; † = Difference from immediately post-exercise; $P \leq 0.05$)

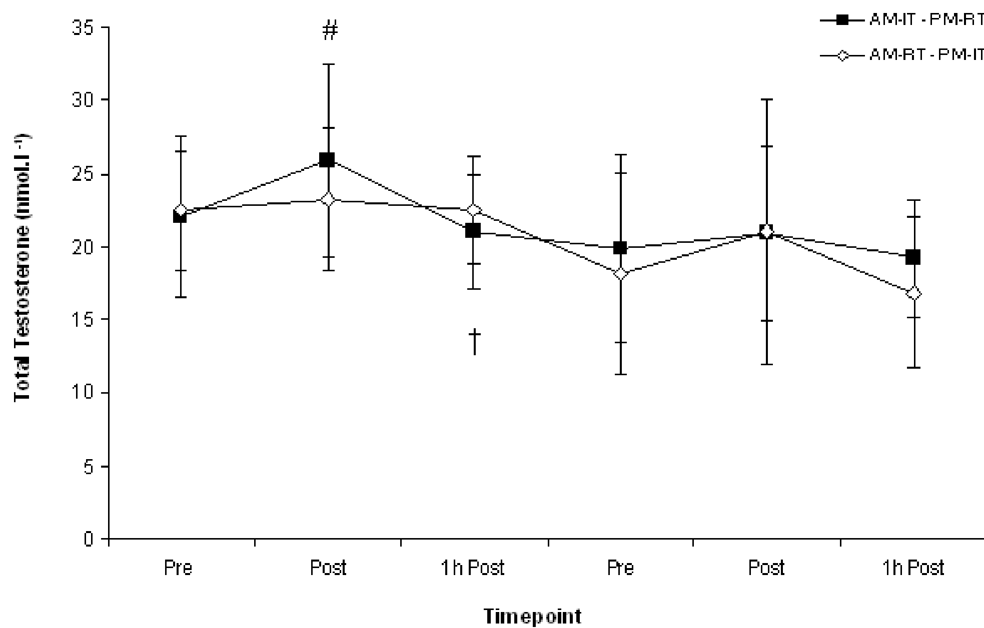

Figure 7.3 Change in total testosterone concentration across trials with two exercise bouts (AM-IT – PM-RT = Morning interval exercise, afternoon resistance exercise. AM-RT – PM-IT = Morning resistance exercise, afternoon interval exercise). (* = Difference between trials; # = Difference from pre-exercise; † = Difference from immediately post-exercise; $P \leq 0.05$)

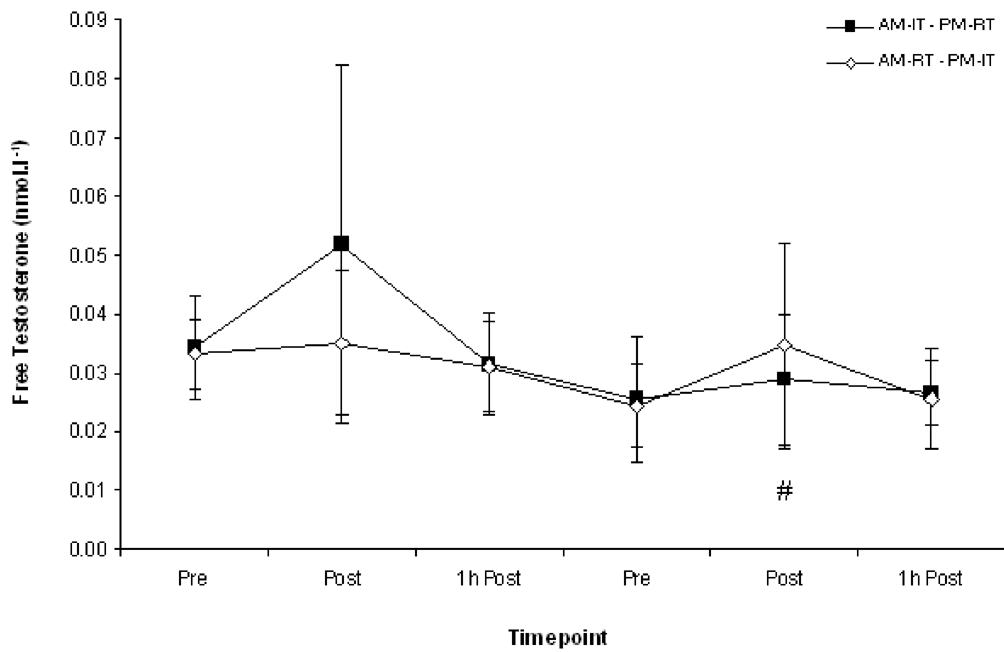

Figure 7.4 Change in free testosterone concentration across trials with two exercise bouts (AM-IT – PM-RT = Morning interval exercise, afternoon strength exercise. AM-RT – PM-IT = Morning resistance exercise, afternoon interval exercise). (* = Difference between trials; # = Difference from pre-exercise; † = Difference from immediately post-exercise; $P \leq 0.05$)

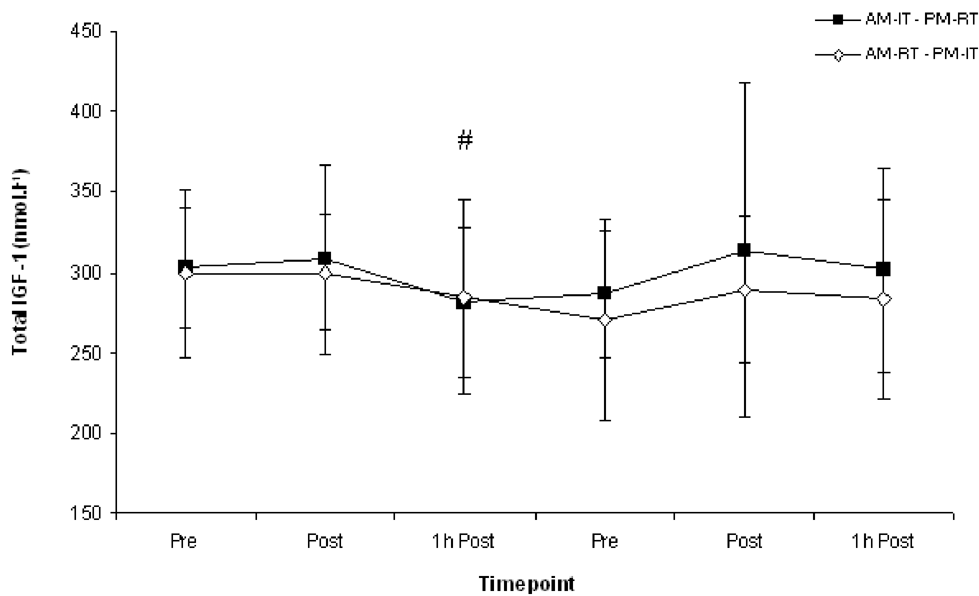

Figure 7.5 Change in total IGF-1 concentration across trials with two exercise bouts (AM-IT – PM-RT = Morning interval exercise, afternoon resistance exercise. AM-RT – PM-IT = Morning resistance exercise, afternoon interval exercise). (* = Difference between trials; # = Difference from pre-exercise; † = Difference from immediately post-exercise; $P \leq 0.05$)

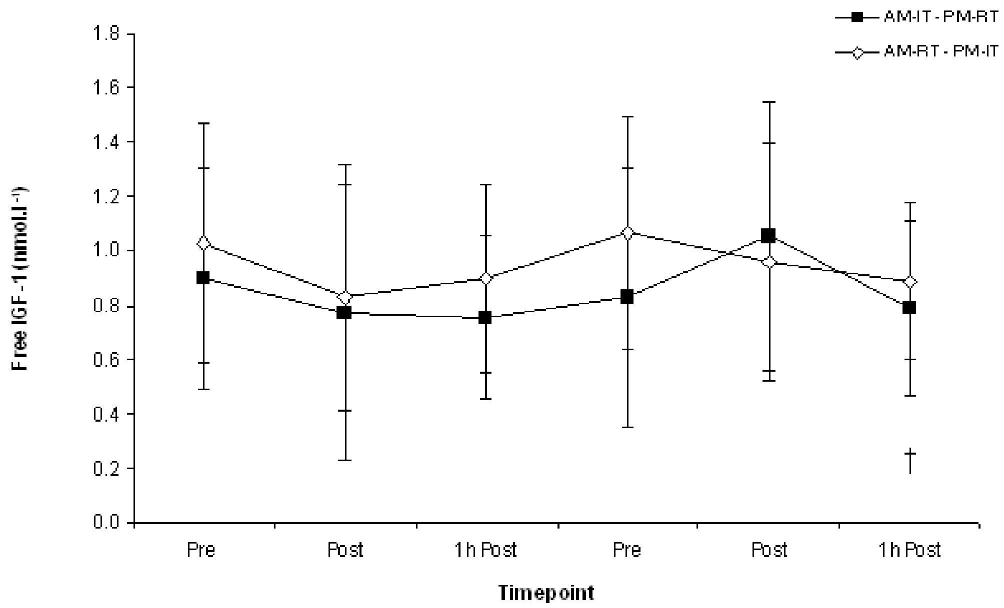

Figure 7.6 Change in free IGF-1 concentration across trials with two exercise bouts (AM-IT – PM-RT = Morning interval exercise, afternoon resistance exercise. AM-RT – PM-IT = Morning resistance exercise, afternoon interval exercise). (* = Difference between trials; # = Difference from pre-exercise; † = Difference from immediately post-exercise; $P \leq 0.05$)

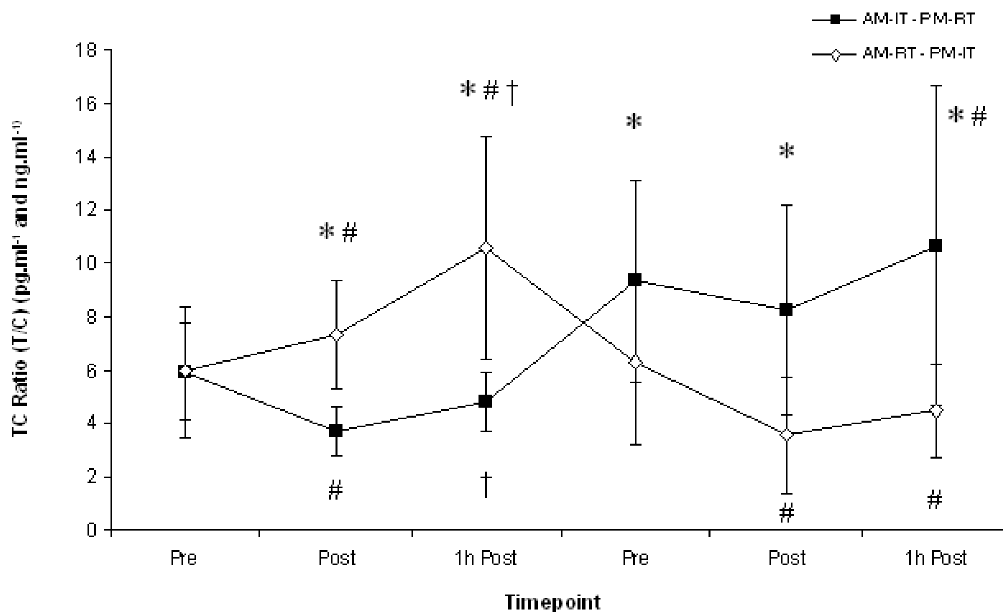

Figure 7.7 Change in TC ratio across trials with two exercise bouts (AM-IT – PM-RT = Morning interval exercise, afternoon resistance exercise. AM-RT – PM-IT = Morning resistance exercise, afternoon interval exercise). (* = Difference between trials; # = Difference from pre-exercise; † = Difference from immediately post-exercise; $P \leq 0.05$)

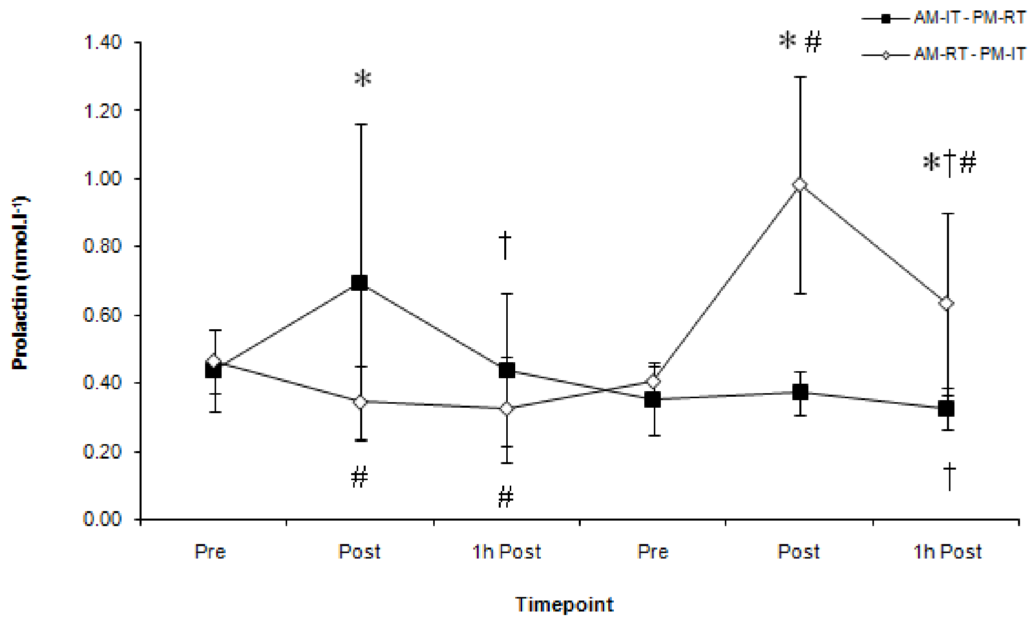

Figure 7.8 Change in prolactin concentration across trials with two exercise bouts (AM-IT – PM-RT = Morning interval exercise, afternoon resistance exercise. AM-RT – PM-IT = Morning resistance exercise, afternoon interval exercise). (* = Difference between trials; # = Difference from pre-exercise; † = Difference from immediately post-exercise; $P \leq 0.05$)

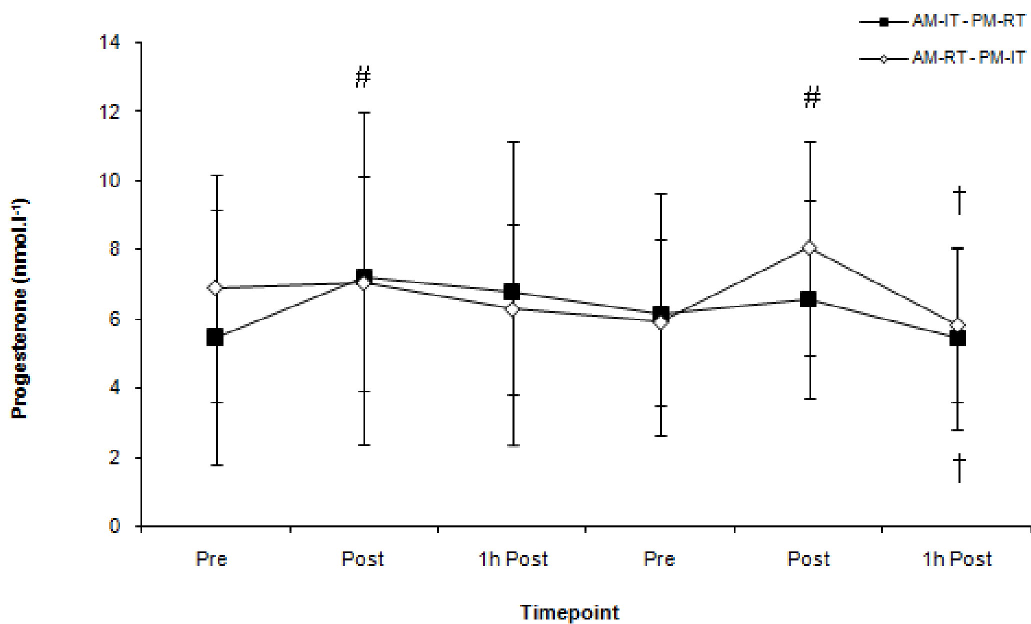

Figure 7.9 Change in progesterone concentration across trials with two exercise bouts (AM-IT – PM-RT = Morning interval exercise, afternoon resistance exercise. AM-RT – PM-IT = Morning resistance exercise, afternoon interval exercise). (* = Difference between trials; # = Difference from pre-exercise; † = Difference from immediately post-exercise; $P \leq 0.05$)

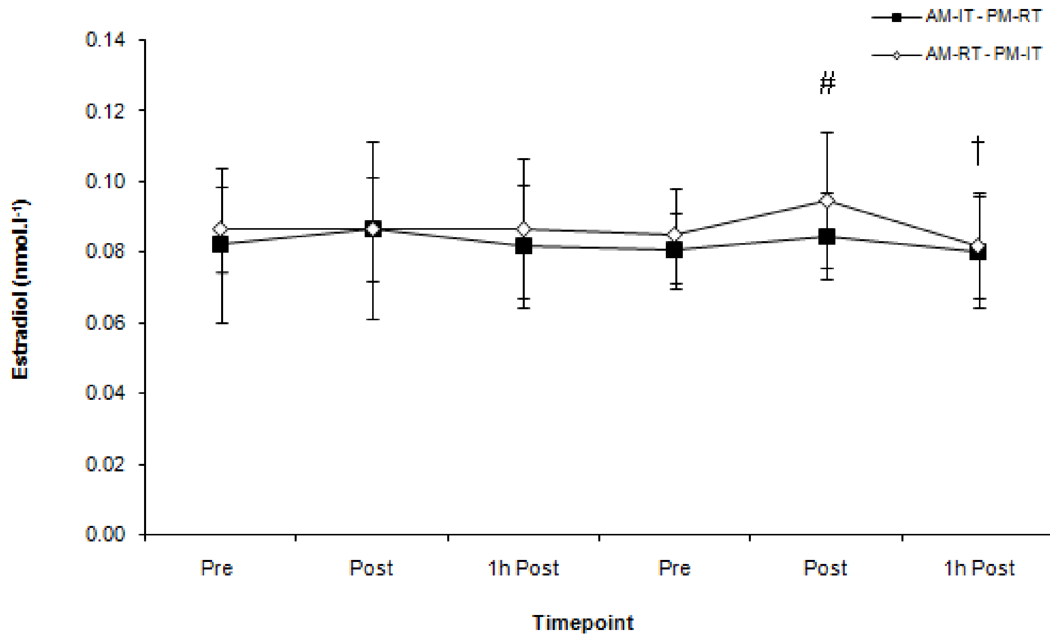

Figure 7.10 Change in estradiol concentration across trials with two exercise bouts (AM-IT – PM-RT = Morning interval exercise, afternoon resistance exercise. AM-RT – PM-IT = Morning resistance exercise, afternoon interval exercise). (* = Difference between trials; # = Difference from pre-exercise; † = Difference from immediately post-exercise; $P \leq 0.05$)

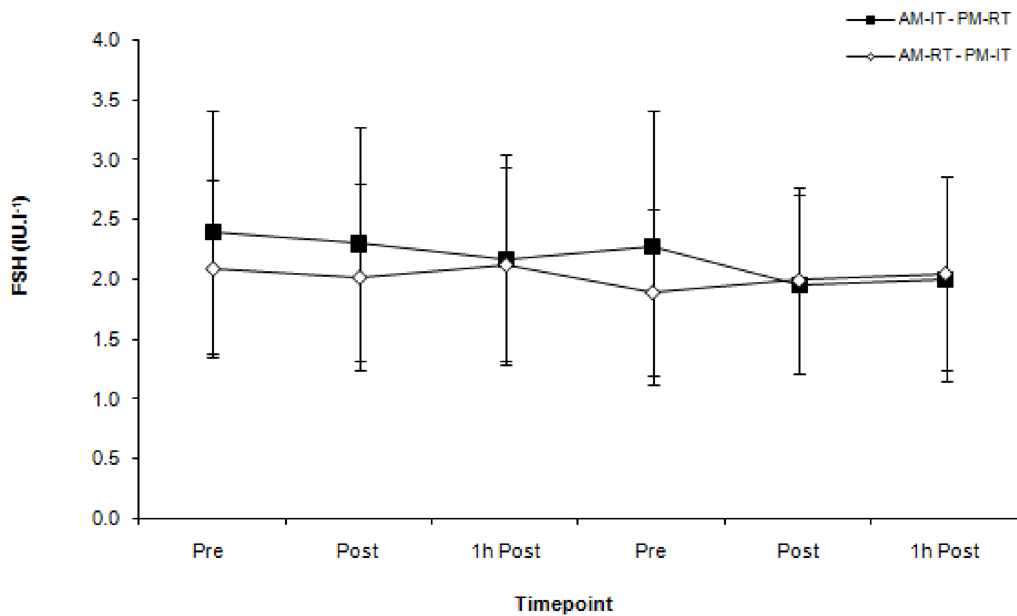

Figure 7.11 Change in FSH concentration across trials with two exercise bouts (AM-IT – PM-RT = Morning interval exercise, afternoon resistance exercise. AM-RT – PM-IT = Morning resistance exercise, afternoon interval exercise). (* = Difference between trials; # = Difference from pre-exercise; † = Difference from immediately post-exercise; $P \leq 0.05$)

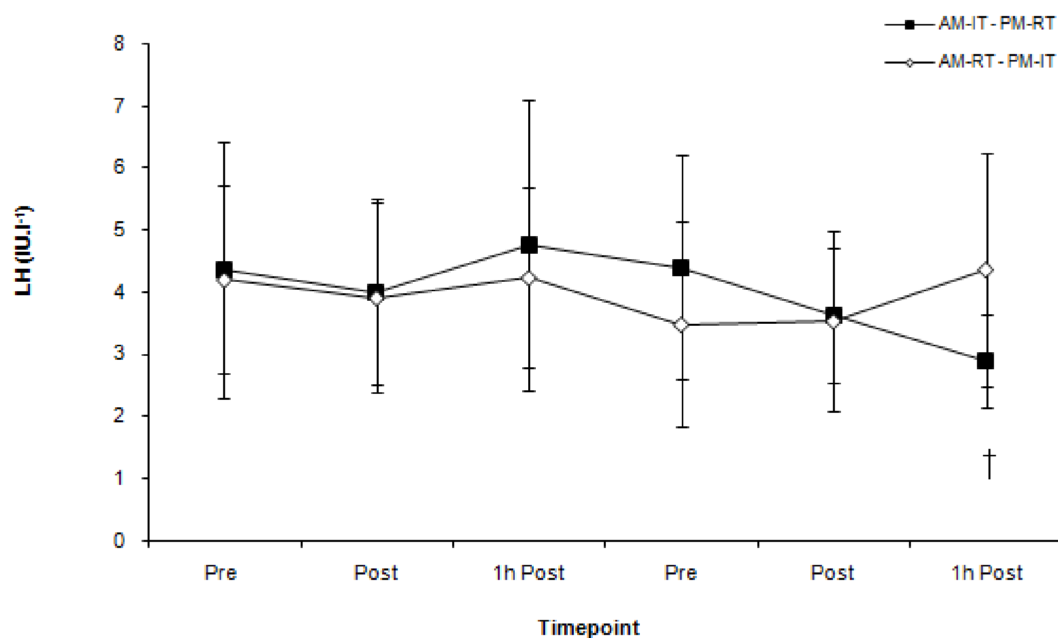

Figure 7.12 Change in LH concentration across trials with two exercise bouts (AM-IT – PM-RT = Morning interval exercise, afternoon resistance exercise. AM-RT – PM-IT = Morning resistance exercise, afternoon interval exercise). (* = Difference between trials; # = Difference from pre-exercise; † = Difference from immediately post-exercise; $P \leq 0.05$)

Table 7.1 IAUC from pre-exercise to 1 h post-exercise for all hormones during both trials. (* = Difference between trials in the response to the same exercise bout type; $P \leq 0.05$).

	AM-IT – PM-RT		AM-RT – PM-IT	
	AM (nmol.l^{-1}.120 min)	**PM (nmol.l^{-1}.120 min)**	**AM (nmol.l^{-1}.120 min)**	**PM (nmol.l^{-1}.120 min)**
Hormone				
Cortisol	18411 ± 8603	2927 ± 4389 *	-8143 ± 6136 *	20374 ± 9987
Testosterone	198 ± 290	43 ± 379	46 ± 105	135 ± 335
Free Testosterone	1.0 ± 1.5	0.2 ± 0.3	0.1 ± 0.7	0.7 ± 0.9
IGF-1	-305 ± 2163	2069 ± 6611	-380 ± 2518	1491 ± 4311
Free IGF-1	-11.8 ± 32.1	12.4 ± 32.2	-16.1 ± 26.1	-11.6 ± 14.6
Prolactin	15.7 ± 32.4 *	0.1 ± 7.2 *	-11.4 ± 7.9 *	41.6 ± 23.1 *
Progesterone	122 ± 119	5 ± 11	-10 ± 135	126 ± 117
Estradiol	0.2 ± 0.2	0.2 ± 0.4	0.0 ± 0.7	0.5 ± 0.7
FSH (Ul.l^{-1})	-12 ± 37	-26 ± 48	-3 ± 25	11 ± 31
LH (Ul.l^{-1})	-8 ± 170	-80 ± 185	-16 ± 122	30 ± 73
TC Ratio	-168 ± 197	-27 ± 279	222 ± 164	-218 ± 191

Table 7.2 Total AUC for all hormones over both trials. (* = Difference between trials; $P \leq 0.05$).

Hormone	AM-IT – PM-RT (nmol.l^{-1}.420 min)	AM-RT – PM-IT (nmol.l^{-1}.420 min)
Cortisol	152357 ± 22991	143413 ± 33461
Testosterone	8959 ± 1902 *	8721 ± 2264 *
Free Testosterone	13.5 ± 4.9	12.6 ± 3.3
IGF-1	123467 ± 20023 *	119365 ± 20911 *
Free IGF-1	351 ± 128	401 ± 149
Prolactin	182 ± 54	199 ± 50
Progesterone	2364 ± 979	3049 ± 1419
Estradiol	34.6 ± 6.2 *	36.5 ± 6.3 *
FSH (Ul.l^{-1})	919 ± 386	847 ± 309
LH (Ul.l^{-1})	1775 ± 423	1629 ± 519
TC Ratio	2909 ± 902	2992 ± 981

Table 7.3 Percentage change in mean concentration from pre-exercise to immediately post-exercise

		Interval Exercise			Resistance Exercise		
Hormone	**Time**	**Pre- (nmol.l^{-1})**	**Post- (nmol.l^{-1})**	**Change (%)**	**Pre- (nmol.l^{-1})**	**Post- (nmol.l^{-1})**	**Change (%)**
Cortisol	AM	390	677	84 ± 45	381	318	-16 ± 13
	PM	305	609	120 ± 72	217	271	25 ± 21
Testosterone	AM	22.1	25.9	19 ± 20	22.5	23.2	3 ± 6
	PM	18.1	21.0	16 ± 24	19.9	20.9	7 ± 18
Free Testos.	AM	0.03	0.05	45 ± 49	0.03	0.04	5 ± 23
	PM	0.02	0.03	39 ± 43	0.03	0.03	13 ± 11
IGF-1	AM	302	307	2 ± 12	299	300	2 ± 15
	PM	270	288	9 ± 18	286	313	9 ± 29
Free IGF-1	AM	0.90	0.77	4 ± 85	1.03	0.83	-15 ± 31
	PM	1.06	0.96	-7 ± 27	0.83	1.05	57 ± 107
Prolactin	AM	0.44	0.70	60 ± 92	0.46	0.36	-26 ± 15
	PM	0.40	0.98	141 ± 58	0.35	0.37	10 ± 23
Progesterone	AM	5.5	7.2	37 ± 25	6.9	7.0	9 ± 23
	PM	5.9	8.0	41 ± 35	6.1	6.6	17 ± 26
Estradiol	AM	0.08	0.09	4 ± 6	0.09	0.09	0.3 ± 8
	PM	0.08	0.09	11 ± 8	0.08	0.08	5 ± 5
FSH (Ul.l^{-1})	AM	2.4	2.3	-3 ± 16	2.1	2.0	-4 ± 12
	PM	1.9	2.0	5 ± 18	2.3	1.9	-9 ± 13
LH (Ul.l^{-1})	AM	4.4	4.0	13 ± 87	4.2	3.9	-3 ± 39
	PM	3.5	3.5	5 ± 19	4.4	3.6	-6 ± 50
TC Ratio	AM	5.9	3.7	-30 ± 29	5.9	7.3	25 ± 21
	PM	6.3	3.6	-42 ± 23	9.3	8.2	-10 ± 30

7.3.5 Individual Responses

7.3.5.1 Interval Exercise:

Responses to interval exercise in the morning and the afternoon demonstrated considerable inter-and intra-individual differences. These differences in response were most marked for total testosterone (Fig. 7.13), free testosterone (Fig. 7.14), progesterone (Fig. 7.15), estradiol (Fig. 7.16), FSH (Fig. 7.17) and LH (Fig. 7.18).

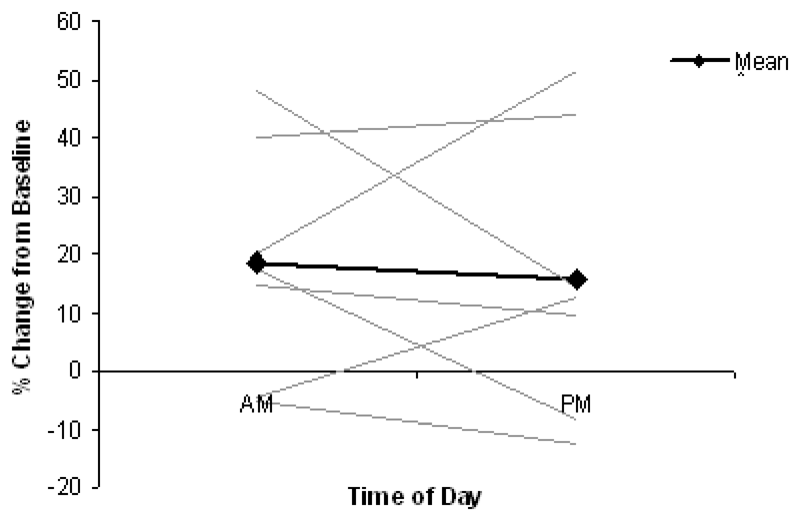

Figure 13 Individual change in total testosterone concentration from pre to immediately post-exercise for interval exercise performed in the morning and in the afternoon.

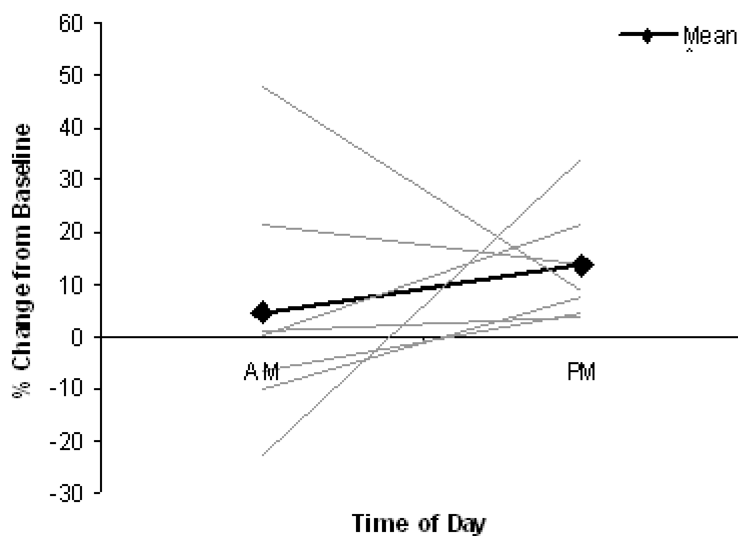

Figure 14 Individual change in free testosterone concentration from pre to immediately post-exercise for interval exercise performed in the morning and in the afternoon.

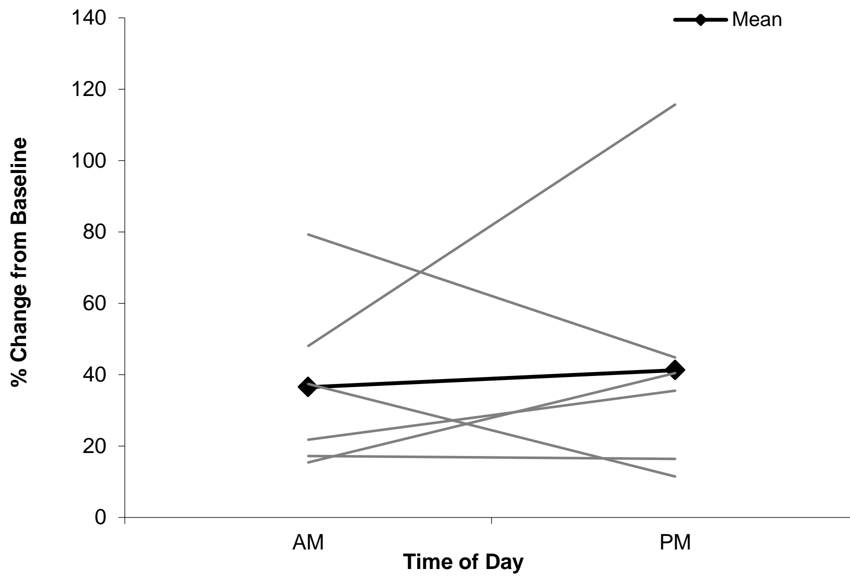

Figure 7.15 Individual change in progesterone concentration from pre to immediately post-exercise for interval exercise performed in the morning and in the afternoon.

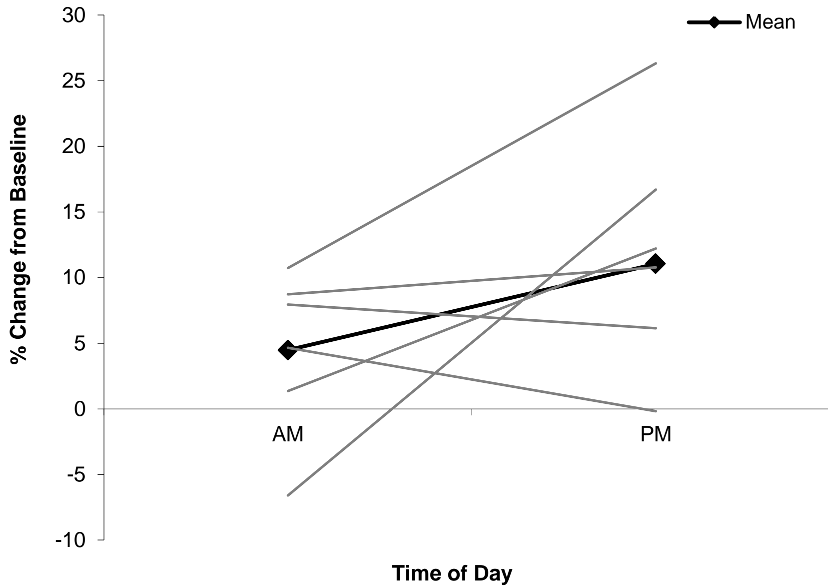

Figure 7.16 Individual change in estradiol concentration from pre to immediately post-exercise for interval exercise performed in the morning and in the afternoon.

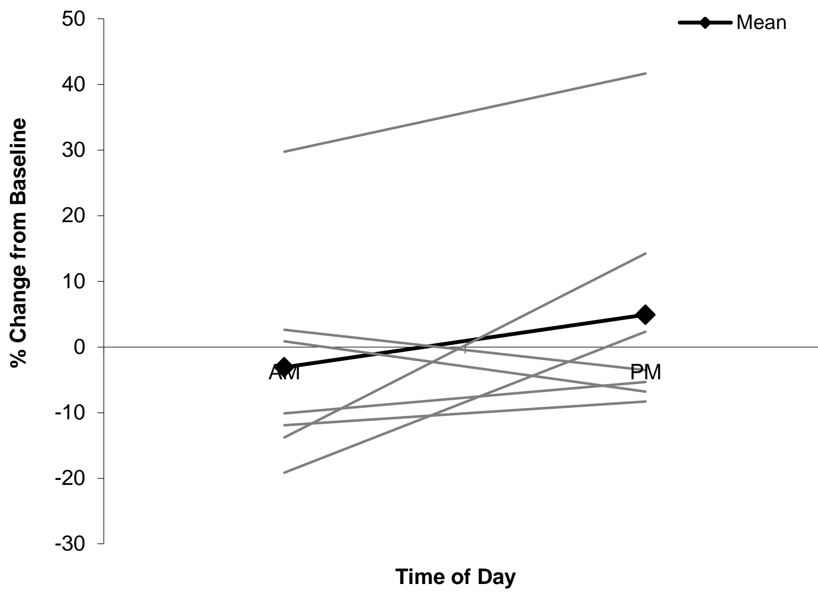

Figure 7.17 Individual change in FSH concentration from pre to immediately post-exercise for interval exercise performed in the morning and in the afternoon.

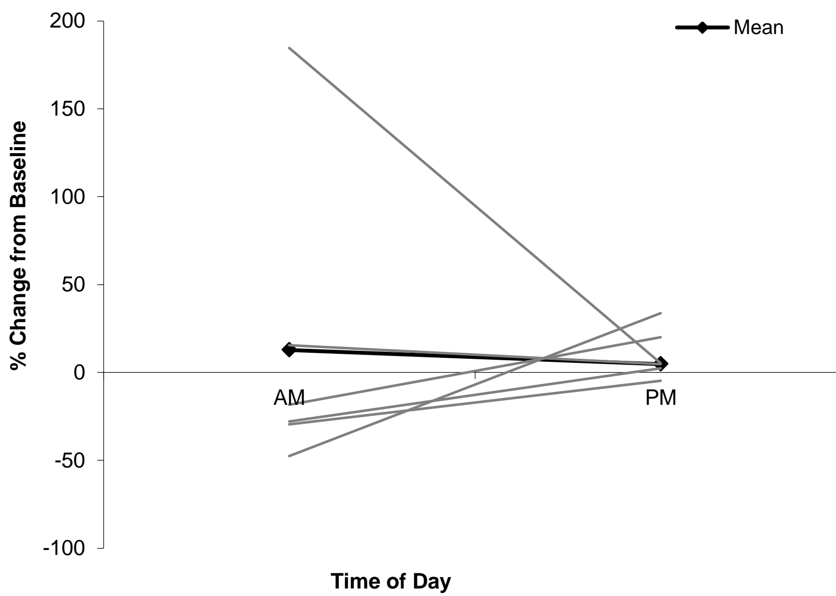

Figure 7.18 Individual change in LH concentration from pre to immediately post-exercise for interval exercise performed in the morning and in the afternoon.

7.3.5.2 Resistance Exercise:

Responses to resistance exercise in the morning and afternoon also demonstrated prominent inter- and intra- individual differences. These differences in response were most marked for total testosterone (Fig. 7.19), free testosterone (Fig. 7.20), total IGF-1 (Fig. 7.21), progesterone (Fig. 7.22). FSH (Fig. 7.23), and LH (Fig. 7.24).

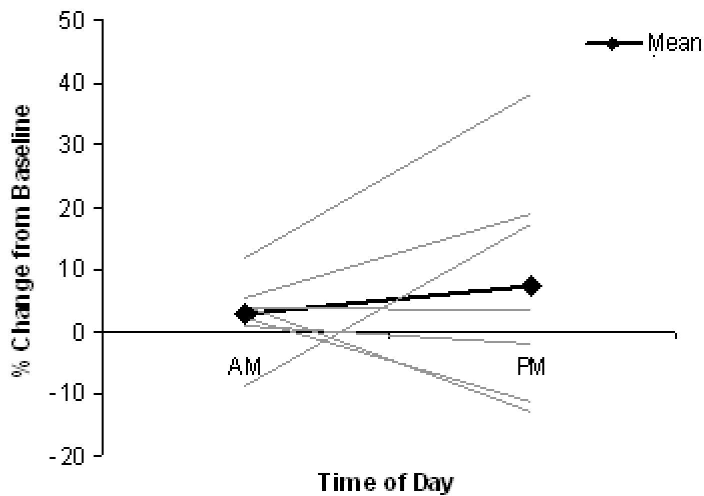

Figure 7.19 Individual change in total testosterone concentration from pre to immediately post-exercise for resistance exercise performed in the morning and in the afternoon.

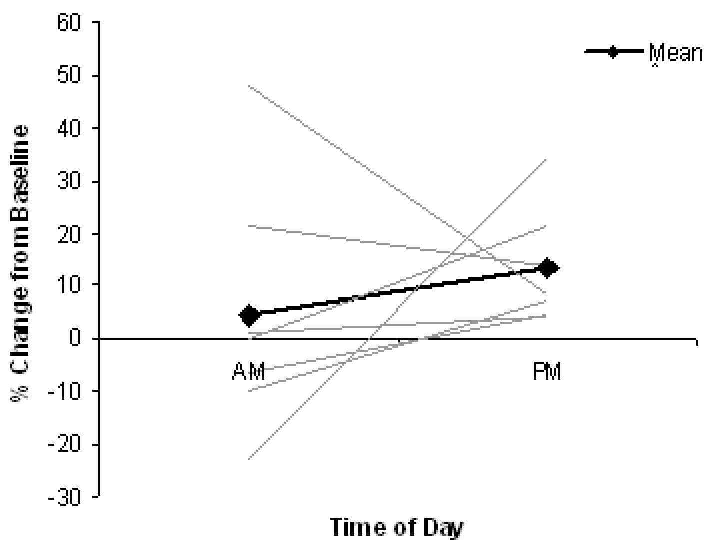

Figure 7.20 Individual change in free testosterone concentration from pre to immediately post-exercise for resistance exercise performed in the morning and in the afternoon.

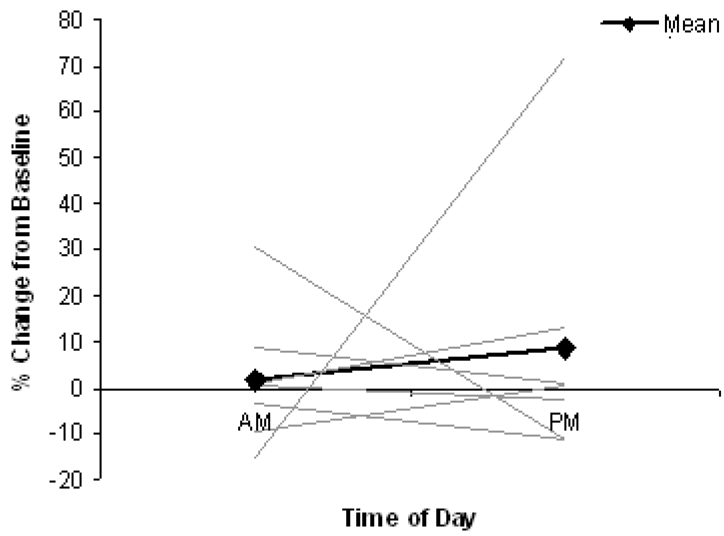

Figure 7.21 Individual change in total IGF-1 concentration from pre to immediately post-exercise for resistance exercise performed in the morning and in the afternoon.

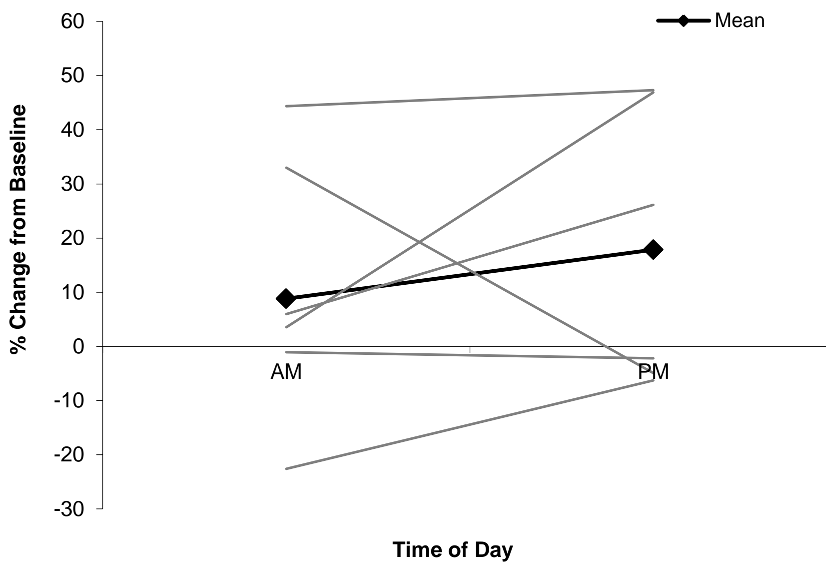

Figure 7.22 Individual change in progesterone concentration from pre to immediately post-exercise for resistance exercise performed in the morning and in the afternoon.

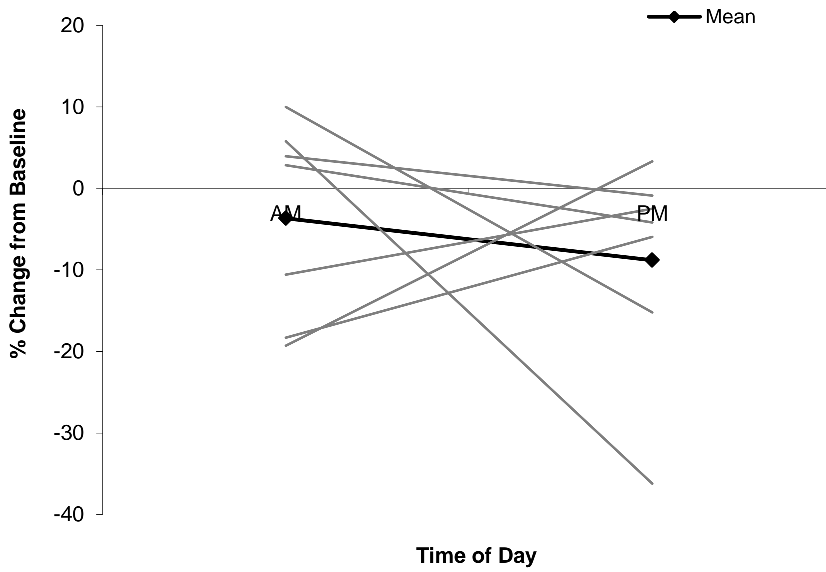

Figure 7.23 Individual change in FSH concentration from pre to immediately post-exercise for resistance exercise performed in the morning and in the afternoon.

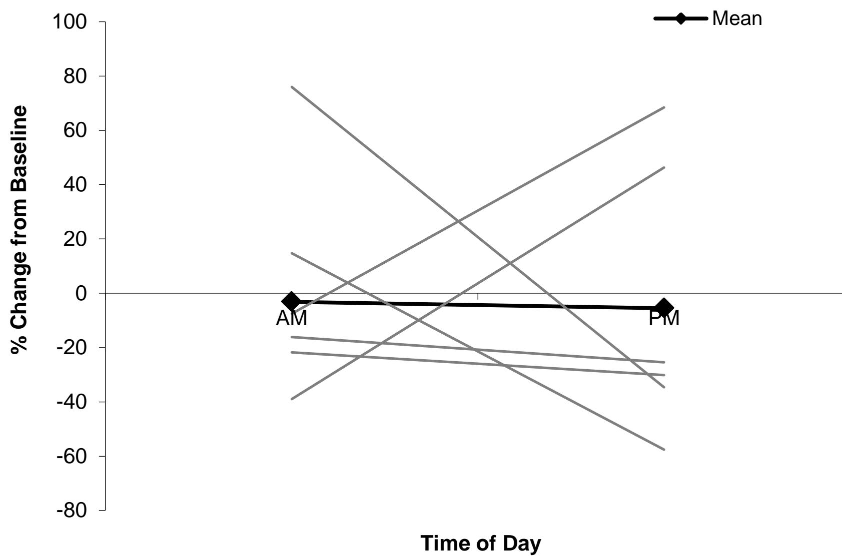

Figure 7.24 Individual change in LH concentration from pre to immediately post-exercise for resistance exercise performed in the morning and in the afternoon.

7.4 Discussion

The main findings of the present investigation demonstrate that interval exercise performed in the morning resulted in significant increases in concentrations of cortisol, total testosterone and progesterone. Resistance exercise performed in the morning elicited a significant increase in the TC ratio while cortisol and prolactin concentrations were significantly lower post-exercise. Where two exercise bouts were completed during the same day, the magnitude of change in hormone concentration in response to resistance exercise in the afternoon following completion of interval exercise in the morning was numerically greater for all hormones in comparison to completion of resistance exercise in the morning, but this was only significant for cortisol and prolactin. However, for cortisol, this may have been influenced by a lower afternoon baseline concentration. The magnitude of change in concentration in response to interval exercise in the afternoon following completion of resistance exercise in the morning was varied, but was significantly greater for prolactin and the TC ratio .

In agreement with the present results, sprint and high intensity interval exercise have previously been shown to stimulate significant increases in concentrations of cortisol, total testosterone, prolactin, estradiol and progesterone (Gray *et al.*, 1993; Vincent *et al.*, 2004; Pullinen *et al.*, 2005; Meckel *et al.*, 2009; Stokes *et al*., 2013). Hypertrophy resistance exercise has consistently been reported as generating the greatest perturbation in hormone concentrations in comparison to other resistance exercise protocols (Kraemer and Ratamess, 2005; Crewther *et al.*, 2006). The hypertrophy resistance exercise protocol employed in the present study was based upon those previously used in the literature (Hakkinen & Pakarinen, 1995; Ratamess *et al.*, 2005).

In agreement with the present observations of the response of hormone concentration to morning resistance exercise, decreased concentrations of cortisol immediately following hypertrophy resistance exercise have been reported previously (Hakkinen and Pakarinen, 1993; Bosco *et al.*, 2000; Beaven *et al.*, 2008a), although this may be a reflection of cortisol concentrations naturally falling during the morning due to circadian variation (Diver *et al.*, 2003). Increases in cortisol concentration have also previously been reported in response to hypertrophy-type resistance exercise (Crewther *et al.*, 2006; Kraemer and Ratamess, 2005), as was seen in the present investigation following completion of resistance exercise in the afternoon after interval exercise in the morning. However, these sessions were not preceded by prior exercise. In contrast to the present data, significant increases in total and free testosterone following hypertrophy resistance exercise have been frequently reported (Ahtiainen *et al.*, 2003; Kraemer and Ratamess, 2005; Crewther *et al.*, 2006). Free testosterone only increased in response to afternoon resistance exercise following morning interval exercise.

Exercise intensity, rest periods, and metabolic stress have been identified as key determinants of the hormonal response to any given exercise session (Schwarz *et al.*, 1996; Kanaley *et al.*, 2001; Tremblay *et al.*, 2005; Crewther *et al.*, 2006; Izquierdo *et al.*, 2009). Lactate concentration has been shown to correlate directly with cortisol concentration following interval exercise (Opaszowski and Busko, 2003; Pullinen *et al.*, 2005) and resistance exercise (Hakkinen and Pakarinen, 1993). Lu *et al.* (1997) suggested a direct stimulatory effect of an increase in lactate concentration on total testosterone concentrations following exercise in rats, affected by lactate increasing testicular cAMP production. As such, the difference in post-exercise lactate

concentration between exercise modes in the present study (6.02 ± 3.11 mmol.l^{-1} for interval exercise; 1.50 ± 0.48 mmol.l^{-1} for resistance exercise; $P < 0.05$) may provide some explanation as to the difference in cortisol, and total and free testosterone concentrations seen between exercise modes. Greater lactate concentrations due to an increased metabolic stress stimulated by shorter rest periods between repetitions and sets during hypertrophy resistance exercise in comparison to maximal strength resistance exercise have been suggested to contribute to increases in response of total testosterone to resistance exercise (Goto *et al.*, 2005). The nature of the equipment used for the resistance exercise bout in the present investigation may have influenced the responses observed. With use of the dynamometer, there is no eccentric phase in each 'lift', with only the concentric phase able to be performed. Kraemer *et al.* (2006) reported no difference in total or free testosterone responses between eccentric and concentric resistance exercise, however, the lack of an eccentric phase in each repetition will decrease the training load and intensity across the session, and also effectively increase 'rest' time following each repetition. These factors are likely to have reduced the lactate response to the resistance exercise bout in the present study and contributed to the absence of a response in total and free testosterone.

Minimal change in concentrations of total and free IGF-1, FSH and LH was seen in response to interval exercise or resistance exercise alone, or when preceded by performance of the opposing trial. With regard to total and free IGF-1 this is in agreement with some previous findings demonstrating no change in total (Stokes *et al.*, 2005; Meckel *et al.,* 2009) or free (Stokes *et al.,* 2010) IGF-1 in response to sprint exercise. An increase in pro-inflammatory cytokines such as IL-6 and TNF-α, that often accompanies high intensity exercise, may attenuate the IGF-1 response (Eliakim

& Nemet, 2010). This cytokine response has been shown to be intensity dependent during both resistance exercise (Helge *et al*., 2003) and running (Scott *et al*., 2011) with sprint exercise causing significant elevation in circulating cytokine concentrations (Bishop *et al.,* 2002; Meckel *et al.,* 2009; Meckel *et al.,* 2011). A lack of total IGF-1 response to resistance exercise has been reported previously (Izquierdo *et al.,* 2009; Dalbo *et al.,* 2011), although an increase has also been reported (Rojas Vega *et al.,* 2010). The differences in findings may result from differences in the exercise intensity and metabolic demand of the protocols used. This is likely in the present investigation given the aforementioned nature of the protocol and equipment employed. The minimal response in FSH and LH to both modes of exercise training observed in the present study has also been reported previously following both moderate and high intensity resistance exercise (Raastad *et al.,* 2000). However, increases in both FSH and LH have been reported following a single 400m run sprint (Slowinska-Lisowska & Maida, 2002). The reasons for the discrepancies between studies are unclear. However, FSH and LH are both pulsatile hormones. Sampling timing and frequency may contribute to the reporting of different hormonal profiles in response to sessions. There was also considerable inter-individual and intra-individual variation seen in concentration and patterns of change of both FSH and LH across the course of the day which may influence mean hormone concentrations.

Contrasting findings have been reported in the literature regarding hormone responses to repeated bouts of exercise. No significant differences were observed in cortisol and free testosterone responses to a resistance exercise session alone or when cycle sprint exercise was performed 180 min or 60 min prior (Goto *et al.*, 2007). However, Ronsen *et al.* (2001) demonstrated a significantly greater increase in cortisol

concentration following a repeated high intensity endurance exercise bout with 3 h recovery. The present results demonstrate an augmented response of cortisol to resistance exercise in the afternoon when preceded by morning interval exercise in comparison to performance of resistance exercise in the morning. However, this may be linked to difference in baseline concentrations between morning and afternoon sessions.

Brandenberger *et al.* (1984) stated that high resting cortisol levels provoked feedback suppression of the cortisol response to a subsequent stimulus. Lower pre-exercise cortisol concentrations have been demonstrated to result in a greater cortisol response to interval exercise in comparison to higher pre-exercise concentrations (Jurimae *et al.*, 2004). Due to the circadian rhythm of cortisol it is possible that this effect would occur as a matter of course across a day. As a result, it could be suggested that response of cortisol to exercise would be lower in the morning than in the afternoon when basal concentrations are higher. Kanaley *et al.* (2001) suggested that the cortisol response to exercise is mediated by circadian rhythm of the hormone, and thus, time of day. However, reported increases in cortisol in relation to 30 min of treadmill exercise were greatest at 2400 than either 0700 or 1900, and greater at 0700 than 1900. The present data demonstrate a significantly greater response of cortisol to afternoon resistance exercise than to resistance exercise performed in the morning. This is in agreement with the results of Ronsen *et al.* (2001) and Goto *et al.* (2007). However, these findings were all observed following a prior exercise bout. Kanaley *et al.* (2001) proposed that prior elevation of cortisol by exercise may result in a rebound suppression of the hormone, reducing concentration further than occurs by circadian decrease alone. This may further explain the lower cortisol concentrations prior to the

second exercise bout, which may in turn explain the larger response seen to the second session.

In addition, following an increase in concentration to the earlier exercise bout, pre-exercise total testosterone concentrations prior to the second bout were significantly lower, and demonstrated a significantly greater increase to the subsequent exercise bout, than during the rest or afternoon exercise only trials (Ronsen *et al.*, 2001). This may suggest that this rebound effect, originally suggested in relation to cortisol (Kanaley *et al.*, 2001), may also apply to other hormones. Indeed, prolactin and estradiol concentration increased significantly in response to afternoon interval training whilst they had not done so in the morning, and there was a significant increase in free testosterone, and non-significant trend for this to apply to total testosterone in relation to afternoon resistance exercise in the present study.

Pre-exercise cortisol concentrations in the afternoon were significantly different between trials following completion of different exercise bouts in the morning. This may suggest that the extent of any rebound suppression effect, and/or augmentation of hormone response to a subsequent training session, may be influenced by the intensity of a preceding exercise stimulus, and the magnitude of the hormone response to this exercise stimulus. This assertion is supported by the findings of Goto *et al.* (2007) who also utilised sprint exercise prior to resistance exercise. This may explain the current trend for the response to resistance exercise to be greater for all hormones when performed in the afternoon following prior interval exercise, and the variation in the response to interval exercise in the afternoon when preceded by a lower magnitude of response and lower intensity of the resistance exercise bout.

It may not be sufficient to consider increases and decreases in individual hormone concentrations at specific time-points alone. In order to establish overall exposure of the tissue to a hormone, it may be more pertinent to consider AUC. In addition, given that hormones operate in concert, The TC ratio may provide additional information as to the balance between protein synthesis and degradation within the tissue (Izquierdo *et al.*, 2001; Urhausen *et al.*, 1995). IAUC for the TC ratio from pre-exercise to 1 h post-exercise was not different between trials for resistance exercise. However, the total IAUC across the entire trial day was significantly greater during the morning interval exercise - afternoon resistance exercise trial. If the TC ratio can indeed be used as an indication of the balance between protein synthesis and degradation, then performing exercise bouts in this order may be more beneficial in increasing muscle protein turnover for subsequent protein synthesis and muscular adaptation. Given earlier discussion of the findings of a rebound suppression apparent in some hormones it would seem prudent to consider TAUC across each trial day. The present data indicate across the entire trial day, TAUC was significantly greater for total testosterone and total IGF-1 during the morning interval exercise – afternoon resistance exercise trial. This may suggest performing exercise sessions in this order could be more beneficial for the goal of increasing muscle mass.

Given the number of near significant result in the present investigation, post-hoc power calculations were performed in order to calculate the required sample size to sufficiently power the study and elicit significance. A minimum sample size of 17 would be required in order to find significance in all results displaying a trend. This reinforces the suggestions that training session order may be an important factor for

consideration if the goal is to foster an endogenous hormonal environment that may be beneficial to muscular adaptation.

Individual variation in hormonal responses in relation to exercise session type and order is evident in the present data. These differences encompassed the magnitude, direction and time course of the responses. This suggests that hormone synthesis, release and metabolism may be different between individuals even in relation to identical exercise stimuli. Bird and Tarpenning (2004) hypothesised that inter-individual differences may reflect differences in entrainment of the circadian pacemaker due to differences in habit forming or conditioning but the authors failed to find a link between routine exercise time and hormonal response to resistance exercise. Training background and status of an individual may influence acute response of hormones to exercise. Increased testosterone secretion in response to exercise has been reported in individuals with a longer training history (Kraemer *et al.*, 1992; Fry *et al.,* 1993). Although, resting levels, and response of testosterone to exercise, have been reported to be blunted in endurance trained males in relation to cortisol (Duclos *et al.*, 1998; Daly *et al.*, 2005), and levels of anabolic hormones have been shown to decline with prolonged endurance training (Consitt *et al.*, 2002). In addition, endurance trained individuals have been shown to demonstrate less pronounced cortisol and total and free testosterone responses to resistance and endurance exercise than resistance trained individuals (Tremblay *et al.*, 2004). Given the varied training background of the participants in the current investigation this may provide some explanation as to the wide variation seen in individual responses; however, there were no significant correlations between individual responses and any of the physiological or performance characteristics obtained from participants (BM,

age, VO_2max, maximum strength, and cycling peak power output – data not shown). Genetic polymorphisms relating to different elements of physical performance have been identified (Macarthur and North, 2005), some of which relate to endocrine and hormonal factors, that may influence the development of strength (Kostek *et al.*, 2005; Lapauw *et al.*, 2007) or exercise economy (Lopez-Alarcon *et al.*, 2007). As such, genetic characteristics may also dictate hormonal response to different exercise stimuli. For example, expression of androgen receptor mRNA, at rest and in response to exercise, has been demonstrated to be correlated with free testosterone concentration and response (Roberts *et al.*, 2009). The factors that may influence the individual nature of these responses require further investigation.

7.5 Conclusion

In conclusion, the present incremental and total area under curve data may indicate that it could be beneficial to perform resistance training in the afternoon preceded by interval training in the morning in order to stimulate a hormonal milieu that may be more conducive to increasing muscle protein turnover and increasing muscle mass. The data also demonstrate an augmented response of cortisol to resistance exercise following earlier interval exercise. These findings may be linked the influence of a prior exercise bout, and its intensity, on the circadian rhythm and feedback control of these hormones. The data presented demonstrate that interval exercise can be used to stimulate a pronounced hormone response, greater than that seen by the resistance exercise protocol employed in this study.

- Chapter 8 -

The Testosterone and Dihydrotestosterone Response to Sprint Interval Exercise in Females

8.1 Introduction

Testosterone concentrations are known to increase in response to certain exercise stimuli (Kraemer & Ratamess, 2005; Crewther *et al*., 2006). These responses have been linked to the acute performance of power and strength based tasks (Cardinale & Stone, 2006; Crewther *et al*., 2012a), as well as chronic adaptation to training (Beaven *et al.,* 2008; Crewther *et al.,* 2011), with suggestions that the response may be related to training status (Kraemer *et al.,* 1992; Fry *et al.,* 1993; Crewther *et al* 2006; Crewther *et al*., 2012). Mechanisms underlying these effects have been suggested to include activation of signalling pathways responsible for accretion of skeletal muscle proteins for hypertrophy via mTor (Ferrando *et al*., 2002; Wu *et al*., 2010), those responsible for mobilisation of energy via glutamine transporter 4 (GLUT 4) (Sato *et al*., 2008), as well as influences on behaviour (Archer, 2006) and cognition (Aleman *et al*., 2004) and control of neuromotor unit excitability (Bonifazi *et al*., 2004). The precise nature of the actions of testosterone remains to be elucidated, particularly given the compelling evidence questioning the influence of physiological concentrations of testosterone on adaptations in muscle size and strength (West *et al.,* 2010; West & Phillips, 2011).

Recently, interest has been raised in the downstream androgen converted from testosterone, dihydrotestosterone (DHT). DHT is formed via the reduction of testosterone by the enzyme 5-alpha-reductase (Thigpen *et al.,* 1993). Circulating

concentrations of DHT are lower than those of other androgens with approximately only five per cent of testosterone thought to be converted to DHT. However, DHT is two to three times more potent than its precursor, demonstrating greater affinity for androgen receptors (Bauer *et al.,* 2000) and not dissociating as readily as testosterone from the androgen-receptor complex (Grino *et al*., 1990). Previously, 5-alpha-reductase was thought not to be present in skeletal muscle, however, a further isoform of the enzyme that is highly expressed in skeletal muscle has recently been reported (Godoy *et al.,* 2011). DHT has been suggested to have both functional and signalling effects in this domain, enhancing force production in fast twitch fibre bundles in mice (Hamdi & Mutungi, 2010) and enhancing genomic signalling that promotes transcriptional processes associated with anabolic effects (Yoshioka *et al.,* 2006).

We have previously demonstrated a simultaneous increase in DHT and total and free testosterone in males in response to high intensity repeated cycle sprint exercise (Smith *et al.,* 2013). The aim of the present investigation was to assess the total testosterone and DHT response to a bout of repeated sprint cycle exercise in females, and secondarily, to assess if the response was related to the training status of participants and performance of the exercise bout.

8.2 Methods

8.2.1 Participants

Ten young, healthy females (age: 24.9 ± 3.0 y; VO_2 max 49.5 ± 9.9 $ml.kg.min^{-1}$) participated in this study. Participants activity levels ranged from recreationally active (1-2 exercise sessions per week) to completing substantial training (10-12 exercise sessions per week). Each participant was briefed regarding the nature of the study and provided written informed consent prior to commencing any testing. This study was approved by the University of Bath Research Ethics Approval Committee for Health (REACH).

8.2.2 Preliminary Measurements

Preliminary tests were conducted in order to determine each participant's maximal oxygen uptake. Participants completed an incremental test to exhaustion on a SRM cycle ergometer (Schoberer Rad Messtechnik, Fuchsend, Germany). The protocol included a 10 min warm-up at a self-selected intensity followed by consecutive 3 min stages, at the end of which the load on the ergometer was increased by 30 W. In the final minute of each stage, a one minute expired air sample was collected and analysed for concentrations of O_2 and CO_2 to assess oxygen uptake. The test continued until volitional exhaustion. Data were used to calculate subsequent work intensities for the main trial. Participants were asked to refrain from strenuous exercise and avoid both alcohol and caffeine consumption during the 24 h prior to the main trial.

8.2.3 Experimental Protocol

At least 48 h after completion of the incremental exercise test, participants completed a bout of repeated sprint interval cycle exercise on the same stationary cycle ergometer. The session consisted of 10 x 30 s sprinting at a target power of 150% Wmax as established during the incremental exercise test, with 90 s recovery (as previously described in Chapter 4). The session was self-paced with real-time graphical and numerical feedback provided on elapsed time, cadence and power. Participants were given verbal encouragement throughout.

8.2.4 Sampling and Analysis

On arrival at the laboratory, a 10 ml pre-exercise blood sample was obtained from a superficial antecubital forearm vein. Further blood samples were obtained immediately post-exercise, and 1 h post-exercise. Samples were dispensed into serum collection tubes (Serum Z/5 ml; Sarstedt, Germany) and left to clot for 15 min before being centrifuged for 10 min at 1,500 *g*. Supernatant was removed and immediately transferred to polypropylene Eppendorf tubes and frozen at -20°C until analysis.

Samples were analysed in duplicate via ELISA for total testosterone, DHT and cortisol using commercially available kits (IBL, Hamburg, Germany). Inclusion of the analysis of free testosterone was originally intended, however, concentrations were undetectable in 50% of participants during pilot work. Combined intra- and inter-assay variation was 4.6% and 5.7% respectively for total testosterone, 6.9% and 8.5% for DHT, and 3.0% and 3.5% for cortisol.

8.2.5 Statistical Analyses

One-way ANOVA was used to investigate differences in hormonal concentrations between different time-points. Subsequent two-tailed paired T-Tests were used to determine the location of any variance. Pearson product moment correlations were performed to assess relationships between performance parameters and hormones concentrations. Analyses were conducted using IBM SPSS Statistics (version 20.0.0; IBM, New York, NY). All data are presented as mean ± SD. Statistical significance was accepted at $P \leq 0.05$.

8.3 Results

8.3.1 Hormone Concentrations

Total testosterone concentrations increased by 19 ± 14% ($P < 0.01$) from pre-exercise to immediately post exercise, as did concentrations of DHT (22 ± 19%; $P < 0.01$) and cortisol (45 ± 47%; $P < 0.01$) (Fig. 8.1 - 8.3) . Concentrations of all hormones had returned to baseline values at 1 h post-exercise. Concentrations of DHT correlated with total testosterone concentrations pre- (r = 0.699; $P < 0.05$), post- (r = 0.709; $P < 0.05$) and 1 h post-exercise (r = 0.687; $P < 0.05$).

8.3.2 Performance Parameters

Mean peak sprinting power was 430 ± 77 W, while mean average sprinting power was 289 ± 53 W. Mean peak sprinting cadence was 113 ± 14 rpm, and mean average sprinting cadence was 100 ± 13 rpm. VO_2max was strongly correlated with average sprinting cadence (r = 0.669; $P < 0.05$) and peak sprinting cadence (r = 0.835; $P < 0.05$). Peak sprinting cadence was very strongly correlated with average sprinting cadence (r = 0.992; $P < 0.01$).

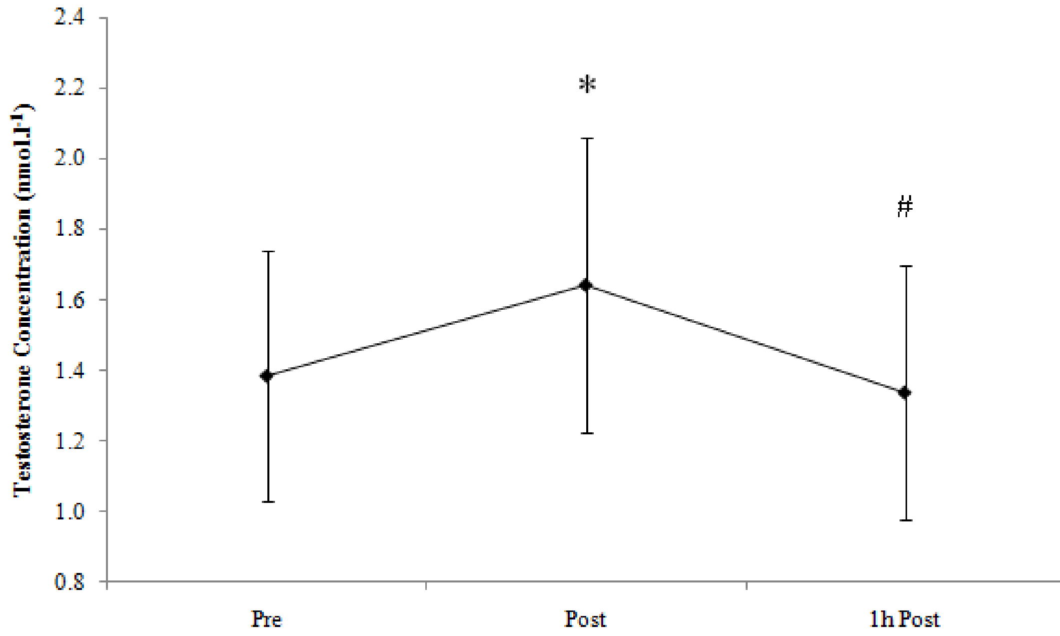

Figure 8.1 Mean testosterone concentration across the trial. * = Significant change from pre-exercise ($P < 0.05$). # = significant change from immediately post-exercise ($P < 0.05$).

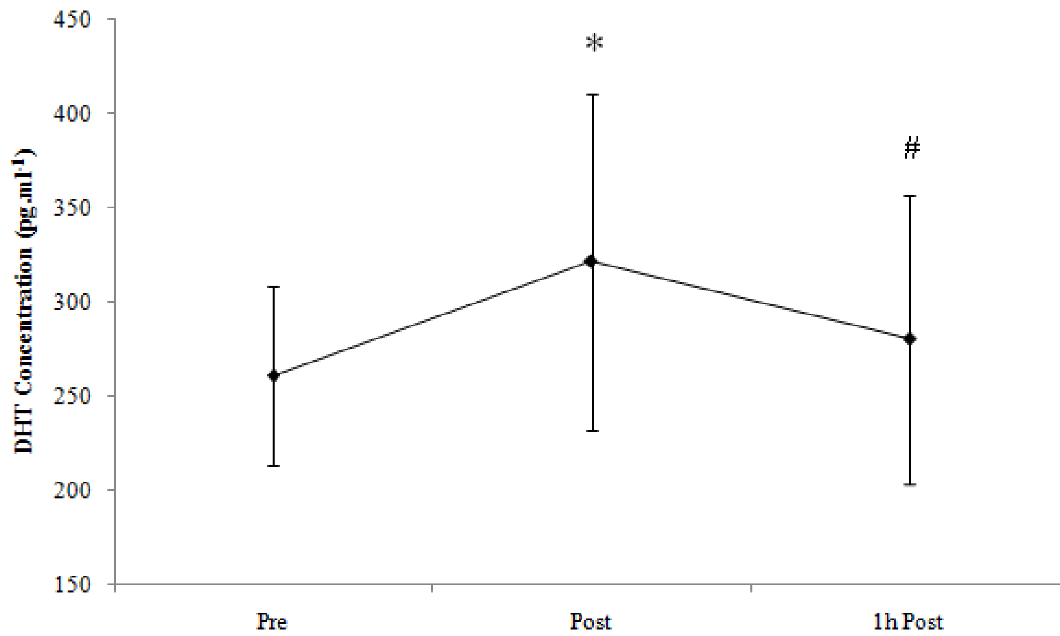

Figure 8.2 Mean DHT concentration across the trial. * = Significant change from pre-exercise. # = significant change from immediately post-exercise ($P < 0.05$).

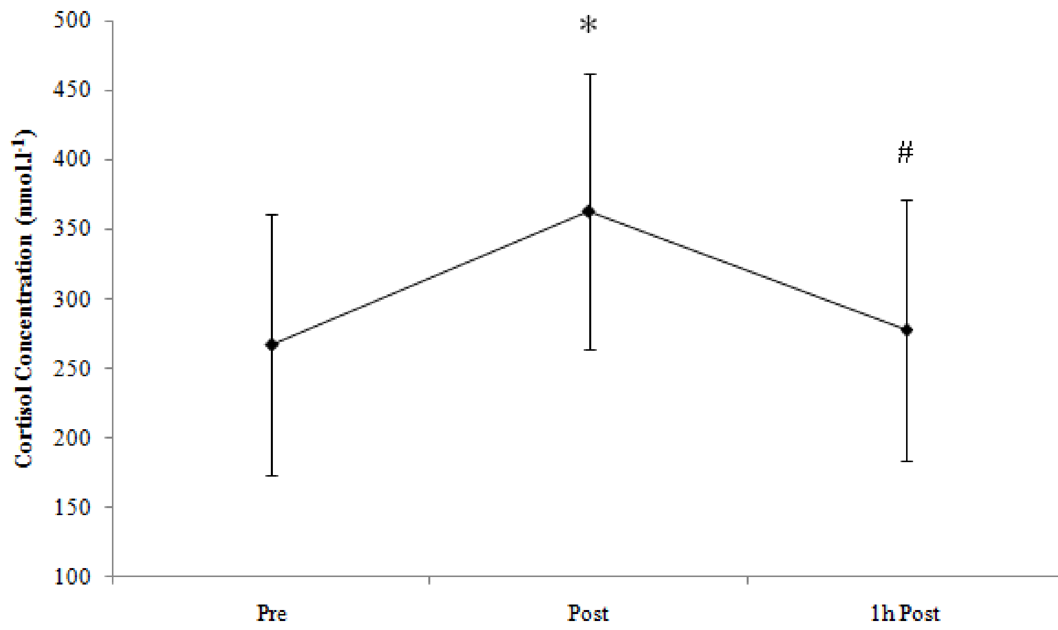

Figure 8.3 Mean cortisol concentration across the trial. * = Significant change from pre-exercise. # = significant change from immediately post-exercise ($P < 0.05$).

8.3.3 Performance and Hormone Concentration Correlations

Post-exercise testosterone concentration was strongly correlated with average sprinting cadence (r = 0.675; $P < 0.05$; Fig. 8.4). Strong correlations were also observed between post-exercise DHT concentration and average sprinting cadence (r = 0.739; $P < 0.05$; Fig. 8.5), and percentage change in DHT concentration from pre-exercise to post-exercise and both peak sprinting cadence (r = 0.828; $P < 0.05$) and average sprinting cadence (r = 0.819; $P < 0.01$). Percentage change in cortisol concentration from pre- to post-exercise was strongly inversely correlated with average sprinting power (r = -0.795, $P < 0.01$) and peak sprinting power (r = -0.662; $P < 0.05$).

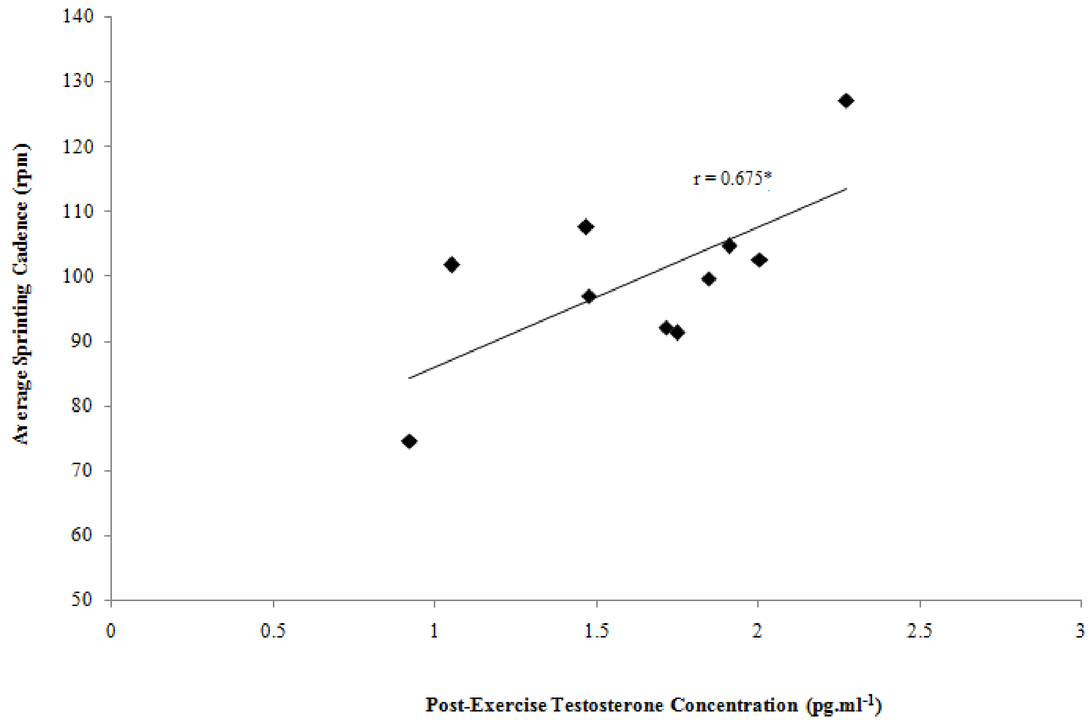

Figure 8.4 Correlation between post-exercise testosterone concentration from pre- to immediately post-exercise and average sprinting cadence (* = P < 0.05).

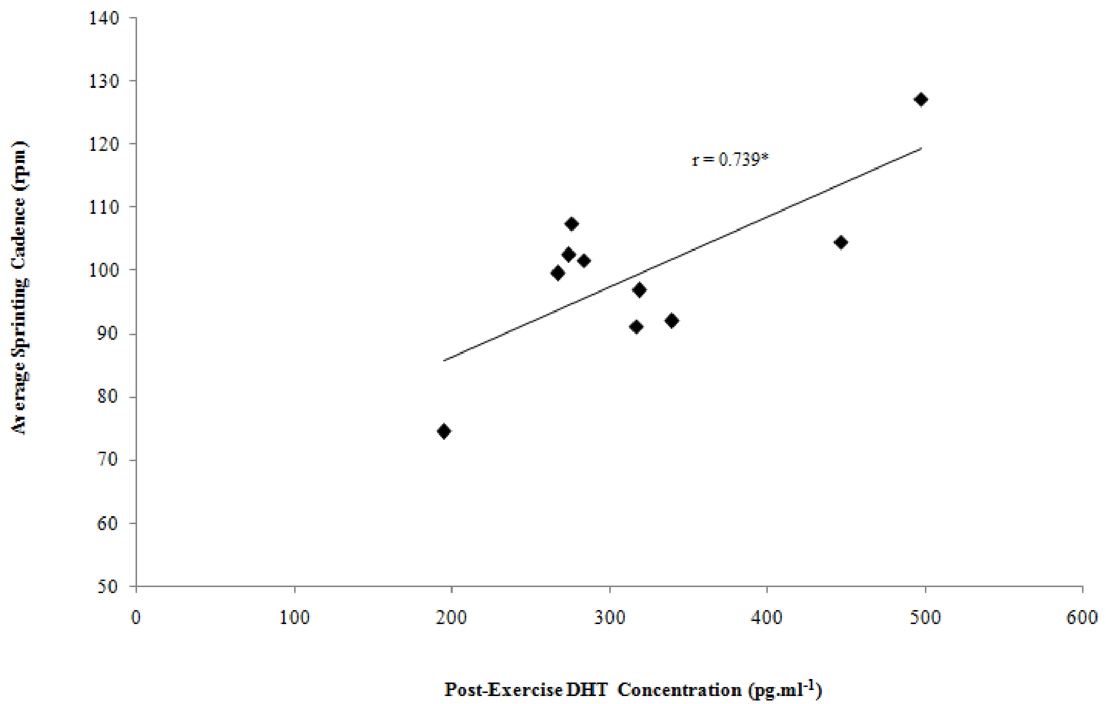

Figure 8.5 Correlation between post-exercise DHT concentration and average sprint power (* = P < 0.01).

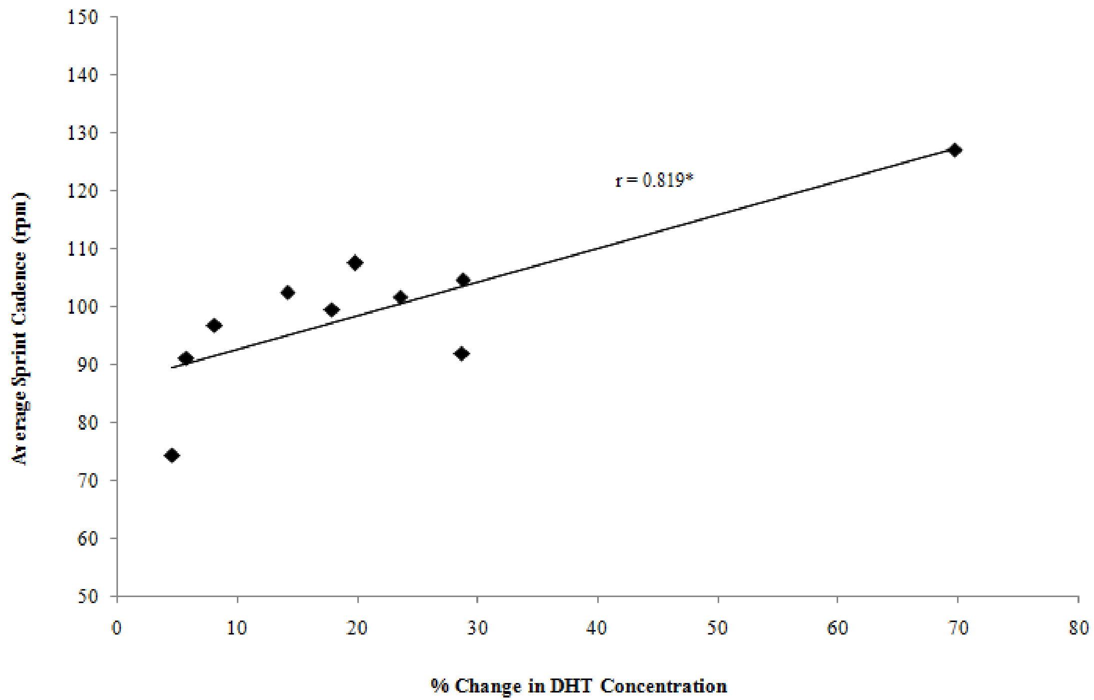

Figure 8.6 Correlation between percentage change in DHT concentration from pre- to post-exercise and average sprint cadence (* = P < 0.01).

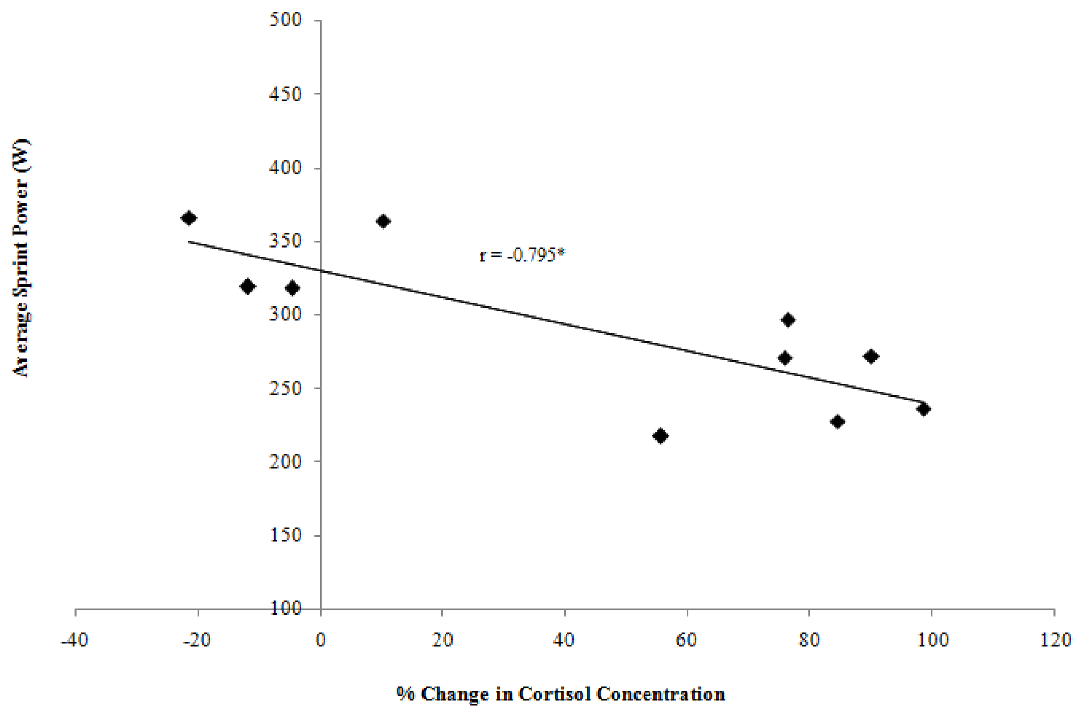

Figure 8.7 Correlation between percentage change in cortisol concentration from pre- to post-exercise and average sprint power (* = P < 0.01).

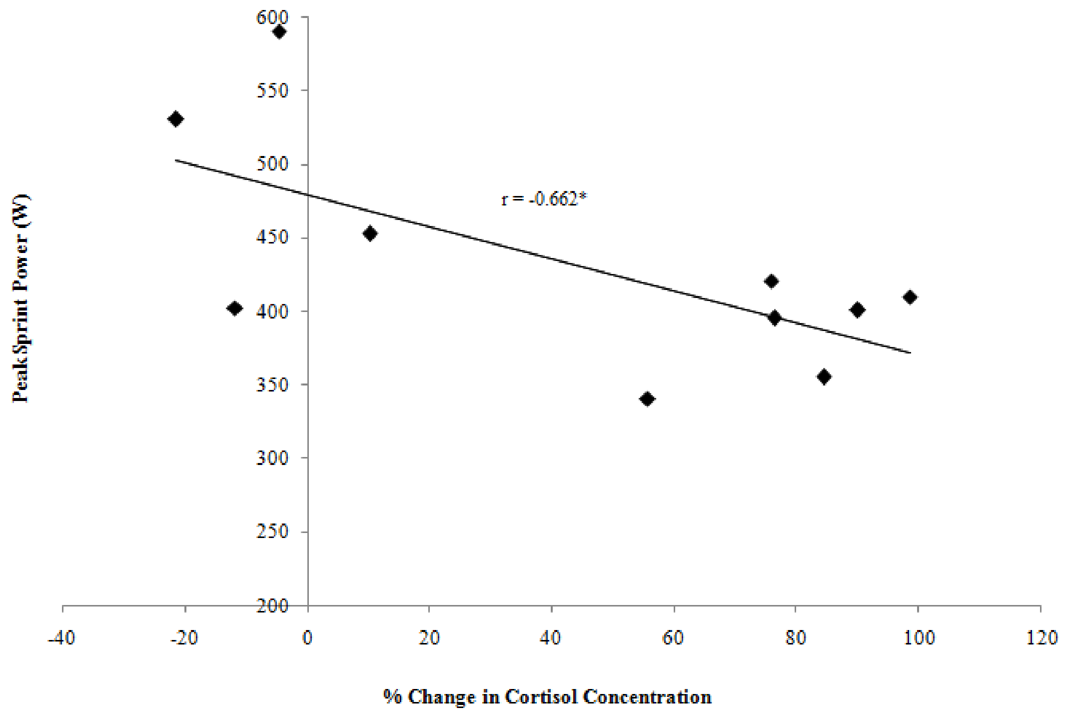

Figure 8.8 Correlation between percentage change in cortisol concentration from pre- to post-exercise and peak sprint power (* = $P < 0.05$).

8.4 Discussion

The present data demonstrate an elevation of circulating serum DHT, total testosterone and cortisol in females immediately following a high intensity repeated sprint cycling protocol. This is the first study to report exercise induced changes in the concentration of DHT in human females. Changes in hormone concentrations were transient, having returned to pre-exercise concentrations by 1 h post-exercise. These data are in agreement with our previous findings of elevations in circulating serum total and free testosterone and DHT in males (Smith *et al.,* 2013), adding a further hormone to the array of those acutely elevated by exercise. Acute elevations of hormones, particularly testosterone, have been shown to play a role in the enhanced performance of strength and power tasks (Cardinale & Stone, 2006; Crewther *et al.,* 2012a), with a role for psychological affect in influencing hormonal concentrations and subsequent performance (Cook & Crewther, 2012). Whether the acute elevations generated by exercise extend to activation of 5α-reductase in skeletal muscle in humans remains to be investigated. However, in rats, exercise has been shown to trigger local muscular conversion of testosterone to DHT (Aizawa *et al*., 2010).

In the present investigation, greater VO_2max was not associated with higher baseline levels of testosterone or DHT, or with greater changes in testosterone or DHT following the high intensity interval exercise bout. This is in contrast to previous data demonstrating chronic elevation in baseline DHT concentration with training (Hawkins *et al*., 2008), and that elevations in testosterone in response to exercise may be dependent upon training status (Crewther *et al.,* 2006; Crewther *et al*., 2012a). However, in females, no difference between trained and untrained individuals has been reported in the response of the androgens estradiol and free testosterone at

exercise intensities from 60 - 80% VO_2max (Keizer *et al.,* 1987). However, large standard deviations generated by a large amount of inter-individual variation in the present data may have contributed to this. In addition, the individuals within this study mostly completed endurance based exercise. It is possible that in a group containing females trained for strength and power would greater responses and magnitudes of change in hormone concentration may have been seen in some of the more well-trained individuals and a correlation may have been seen. Indeed, evidence has shown greater testosterone responses to exercise in strength based males (Crewther *et al*., 2012a; 2012b) and endurance training has been shown to blunt resting androgen concentrations and androgen response to exercise (Izquierdo *et al.,* 2004; Tremblay *et al.,* 2004). Further research may consider assessing the response between un-trained and highly trained individuals of both endurance and strength and power backgrounds.

In the females participating in the present investigation, a strong association was found between percentage change in DHT concentration and peak and average sprinting cadence across the 30 s sprints. This association supports the possibility that the change in DHT concentration may be related to composition and proportions of different muscle fibre types in the active muscle. The proportion of fast twitch fibres in the vastus lateralis of trained cyclists has previously been shown to correlate with optimal sprinting cadence, and maximal sprinting power output (Hautier *et al.,* 1996), while DHT has also been shown to enhance power capability and contractile function only in fast twitch muscle fibres in rodents (Hamdi & Mutungi, 2010). In addition, DHT has also been shown to increase signalling of factors involved in calcium cycling and production of ATP in rodent muscle (Yoshioka *et al.,* 2006), as well as

enhancing glucose metabolism via increasing protein expression and translocation of GLUT-4 (Sato *et al.,* 2008), which may allow greater utilisation of fast twitch fibres through meeting the increased metabolic demand.

Interestingly, and in contrast to our findings in males (Smith *et al.,* 2013) there was no association between percentage change in testosterone concentration and average or peak cadence, or with percentage change in DHT concentration. Synthesis of DHT can however occur in a testosterone independent manner from dehydroepiandrosterone (DHEA) in peripheral intracrine tissues (Luu-The & Labrie, 2010). Given that females have an approximately 10 fold lower concentration of circulating testosterone than males (Vingren *et al.,* 2010), it is possible that production of DHT occurs more readily via testosterone independent pathways. Indeed, in post-menopausal women, generation of the majority of androgens occurs in this manner (Luu-The & Labrie, 2010). In addition, DHT can also be synthesised via a pathway independent of both testosterone and DHEA from conversion of progesterone to androstenedione and then to DHT (Yarrow *et al.,* 2012). This raises the possibility that synthesis of DHT in response to exercise stimuli may be influenced by menstrual cycle phase, and also by the use of oral contraceptives. In the present investigation, menstrual cycle phase and oral contraceptive use was not controlled for and may explain some of the variation seen, and does introduce a considerable limitation to the interpretation of the findings. Previous research has demonstrated a suppression of circulating levels of total and free testosterone, and an increase in circulating SHBG concentrations with use of the oral contraceptive pil, with no effect of different doses or type of estrogen and progestins (Zimmerman *et al.,* 2014). In pre-menopausal women, not using the contraceptive pill, it has been

shown that serum total testosterone, free testosterone, estradiol and SHBG levels peaked at mid-cycle and remained higher in the mid-luteal phase of the cycle, whereas DHT did not change (Rothman *et al.,* 2011). However, evidence also demonstrates that these fluctuations are not large, but that increase in progesterone post-ovulation may result in preferential 5-alpha-reduction of progesterone over testosterone for formation of DHT (Mertens *et al.,* 2001). Further research could consider assessing the DHT response to exercise at different phases of the menstrual cycle alongside responses and concentrations of progesterone and testosterone, and associated strength and power performance.

As a caveat, it should be mentioned that the changes observed in hormone concentrations in the present investigation may also be reflective of a change in plasma volume. It has previously been shown that high intensity sprint exercise can result in a 19% decrease in plasma volume in males (Bloomer & Farney, 2013), and relative increases in total and free testosterone concentrations with exercise have been shown to be no longer evident following adjustment for plasma volume change (White *et al.,* 2002). The raises the possibility that the changes observed in were actually smaller in magnitude, or that no change in the absolute amount of hormone in the circulation occurred which would be suggest the exercise stimulus did not stimulate secretion of the hormones assessed.

8.5 Conclusion

The present data demonstrate that repeated sprint cycle exercise elicits a robust increase in circulating concentrations of testosterone, DHT and cortisol in healthy females. The magnitude of the DHT response appears to be related to sprinting cadence, and is not associated with testosterone response. This may suggest that circulating DHT is synthesised via testosterone-independent pathways in females in response to exercise.

- Chapter 9 -

Hormone, Performance and Affect Responses to Repeated Sprint Cycle Exercise Following Pre-fatigue

9.1 Introduction

Exercise elicits acute changes in circulating hormone concentrations (Kraemer & Ratamess, 2005; Crewther *et al*., 2006; Smith *et al.,* 2013; Stokes *et al.,* 2013), with the magnitude and direction of change dependent upon workout content and design (Crewther *et al.,* 2006). The testosterone and cortisol response to a training session has also been shown to be influenced by psychological and cognitive state and associated with subsequent performance (Cook & Crewther, 2012 a & b), while increased concentrations of testosterone have been shown to improve cognitive function (Aleman *et al.,* 2004), as well as increase aggression (Archer, 2004). In addition, concentrations of testosterone have been associated with the performance of power and strength tasks (Cardinale & Stone, 2006; Crewther et al., 2012a, 2012b). Increased

The response of testosterone and DHT to a repeated sprint cycling exercise bout in trained men has previously been reported (Smith *et al.,* 2013), demonstrating increases in both testosterone and DHT concentrations from pre- to post-exercise, echoing the findings shown for concentrations of total testosterone and cortisol shown in Chapter 7. The acute hormone response to an exercise session has been demonstrated to be influenced by a prior exercise bout (Ronsen *et al.,* 2001; Viru *et al., 2001*; Goto *et al.,* 2007), and has been demonstrated in an earlier in Chapter 7. The changes in response have been varied, and suggested to be related to rebound

suppression or augmentation (Brandenberger *et al.,* 1984; Kanaley *et al.,* 2001). In addition, increases in metabolic stress that have been reported to contribute to increases in the total testosterone (Lu *et al.,* 1997) and cortisol concentrations (Pullinen *et al.,* 2005) during exercise.

Recently, links have been reported between endogenous hormone concentrations and elements of athletic behaviour associated with confidence and motivation to compete (Cook & Crewther, 2012a). Changes in mood state have been shown to influence hormone concentration and performance. Increases in endogenous testosterone have been associated with improved physical performance and skill execution, with increased cortisol related to decreased performance (Cook & Crewther, 2012a; 2012b).

The aim of the present investigation was to assess the hormonal response, performance, and psychological affect to a repeated sprint cycle session when preceded by a 20 min high intensity period of cycling designed to induce fatigue on two occasions. Completion of both trials was completed in a group setting, and as such, it was intended that the performance of an intense, unfamiliar protocol in this environment would be inherently more stressful than on the second occasion; and, thus by repeating the protocol, a dual aim was to assess whether any variation in mood, hormone response or performance between trials was linked.

9.2 Methods

9.2.1 Participants

Fourteen, well-trained male cyclists and triathletes (age: 32 ± 7 y; body mass: 77.8 ± 9.4 kg) participated in this study. All participants completed regular cycling training. Each participant was briefed regarding the nature of the study and provided written informed consent prior to commencing any testing. This study was approved by the University of Bath Research Ethics Committee.

9.2.2 Preliminary Measurements

Preliminary tests were conducted in order to determine each participant's average power output for a maximal 20 min time-trial (TT) effort. Participants completed the test on a Wattbike cycle ergometer (Wattbike Ltd, Nottingham, UK). The protocol included a warm-up at a self-selected intensity followed by a short break and then the 20 min maximal effort TT. Data were used to calculate subsequent target work-loads for the main trials. Participants were asked to refrain from strenuous exercise and avoid both alcohol and caffeine consumption during the 24 h prior to main trials.

9.2.3 Experimental Protocol

One week after completion of the maximal 20 min TT, participants completed the first of the two main trials. The trial consisted of a 20 min effort at a target power of 80% of the average power obtained during the maximal 20 min TT, followed by a 5 min break, before completion of a bout of repeated sprint interval cycle exercise consisting of 10 x 30 s sprinting, with 90 s recovery. The session was self-paced with real-time numerical feedback provided on elapsed time, cadence and power. Participants were given verbal encouragement at specific time-points throughout the trial. The same

protocol was repeated for the second main trial one week later. Participants were permitted to drink water *ad libitum* throughout the trials. Trials were completed in a group setting as a group of six and a group of eight. A trial timeline schematic is displayed below (Fig. 9.1).

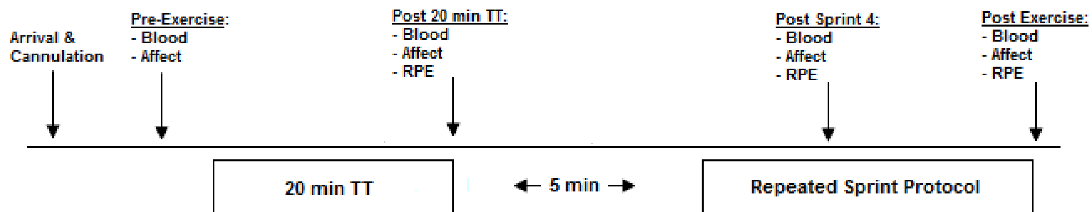

Figure 9.1 Timeline schematic displaying the exercise and sampling procedures for both main trials

9.2.4 *Sampling and Analysis*

On arrival at the laboratory, an indwelling cannula was fitted into an antecubital forearm vein. A pre-exercise 5 ml blood sample was drawn immediately. Further blood samples were obtained following the 20 min effort, in the 90 s recovery period following the fourth sprint, and following the tenth sprint at the end of the trial. Ratings of perceived exertion (RPE) and measures of psychological affect were obtained at the same time-points. Blood samples were dispensed into plasma collection tubes (Plasma EDTA/5 ml; Sarstedt, Sarstedt, Germany) and centrifuged immediately for 10 min at 1,500 *g*. Supernatant was removed and transferred to polypropylene Eppendorf tubes and frozen at -80°C until analysis.

Samples were analysed in duplicate via ELISA for total testosterone, DHT and cortisol using commercially available kits (IBL, Hamburg, Germany). Combined

intra- and inter-assay variation was 4.6% and 5.7% respectively for total testosterone, 6.9% and 8.5% respectively for DHT, and 3.0% and 3.5% for cortisol.

9.2.5 Statistical Analyses

Two-way repeated measures ANOVA was used to investigate differences between trial and time-point for measures of RPE, psychological affect, performance parameters and hormonal concentrations between different time-points and trials. Subsequent two-tailed paired T-tests, with post hoc Bonferroni correction, were carried out to determine the location of any variance. Pearson product moment correlations were performed to assess relationships between variables. All analyses were conducted using IBM SPSS Statistics (version 21.0.0; IBM, New York, NY). All data are presented as mean ± SD. Significance was accepted at $P \leq 0.05$.

9.3 Results

9.3.1 Performance Parameters

9.3.1.1 20 min TT: There were no significant differences between trials in terms of average power output, cadence, or pacing during the 20 min effort with average power not significantly different between each 5 min period of the 20 min effort (Table 9.1).

Table 9.1 Average power during each 5 min period of 20 min pre-fatigue TT effort

	Average Power (W)			
	5 min	**10 min**	**15 min**	**20 min**
Trial 1	233 ± 39	233 ± 39	232 ± 40	232 ± 40
Trial 2	232 ± 42	232 ± 41	232 ± 41	232 ± 41

9.3.1.2 Repeated Sprint Bout:

A significant interaction effect was observed for peak cadence (P = 0.019) with peak cadence for sprints 1 and 2 higher in the second trial than the first ($P < 0.05$) (Fig. 9.4), with a tendency for average cadence to also be higher for these sprints, although this did not reach significance ($P = 0.07$ and $P = 0.06$ for sprints 1 and 2 respectively) (Fig. 9.5).

For the repeated sprint bout, a trend was apparent for peak sprint power to be different between trials (P = 0.07), with peak power higher for sprint 3 in trial 2 in comparison to trial 1 ($P = 0.04$) (Fig. 9.2), however this did not influence average power for those sprints and no difference between trials was found for average sprint power (Fig. 9.3).

On average, the lowest average power for a sprint in the repeated sprint bout was 19 ± 11 % lower than the highest average power. Sprint 1 was most frequently the sprint on which highest average power was obtained, with lowest average power tending to occur at sprint 8-9.

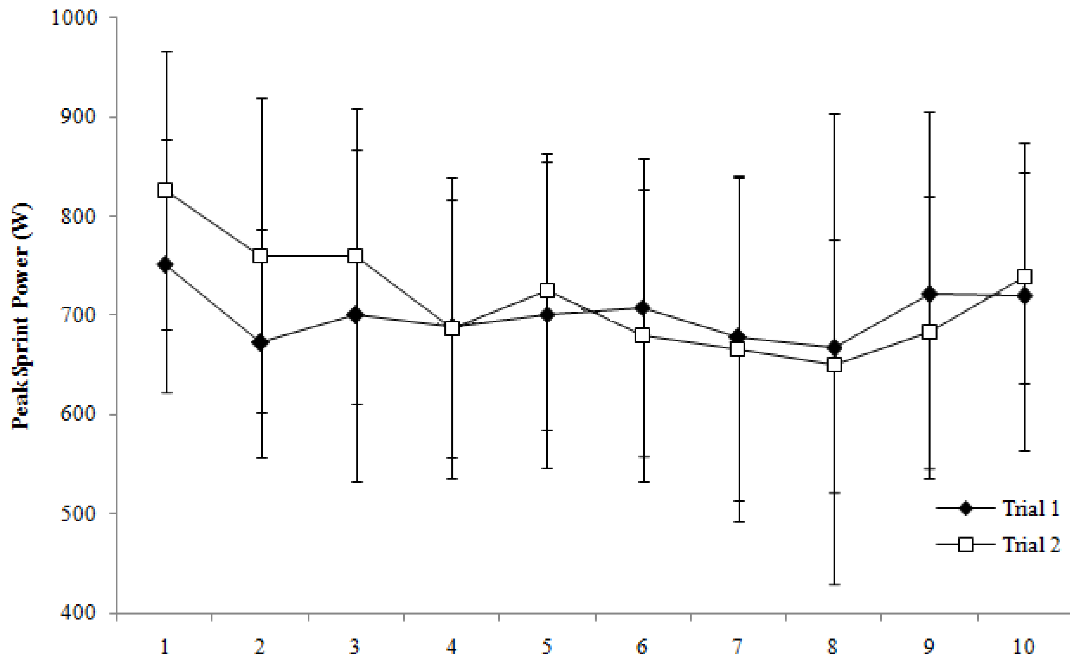

Figure 9.2 Peak sprint power for each sprint during both trials.

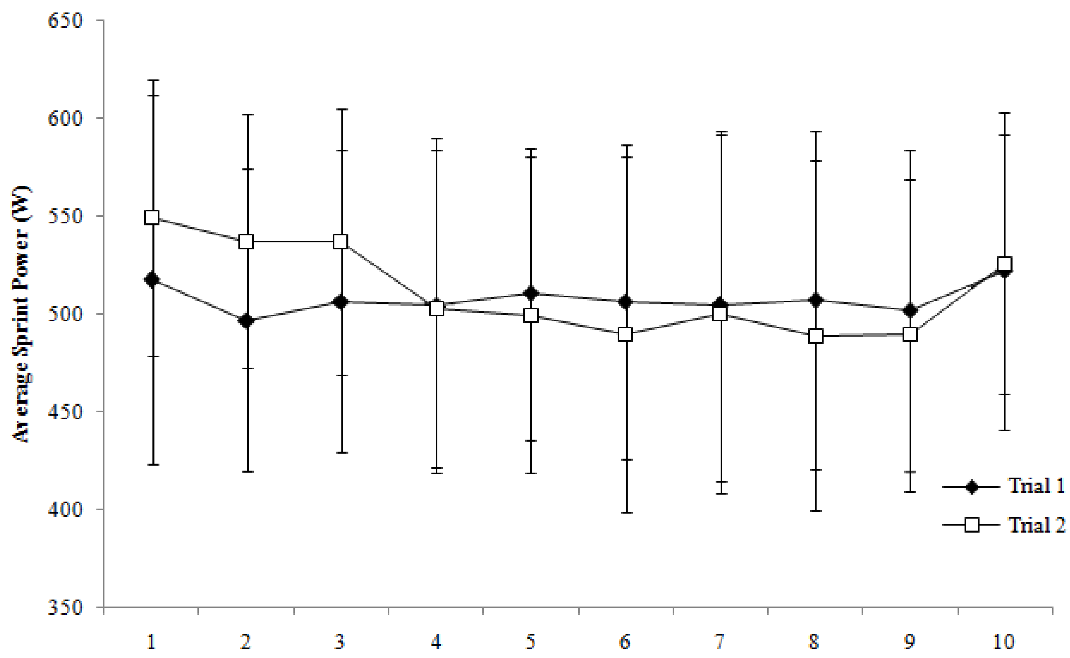

Figure 9.3 Average sprint power for each sprint during both trials.

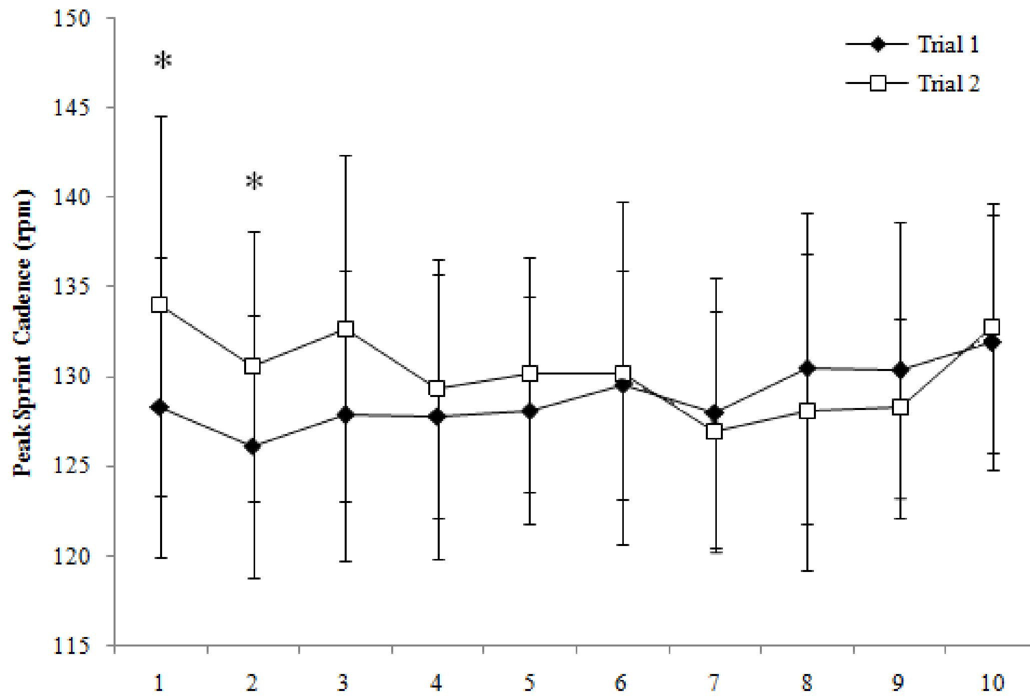

Figure 9.4 Peak sprinting cadence for each sprint during both trials. (* = Difference between trials; $P \leq 0.05$)

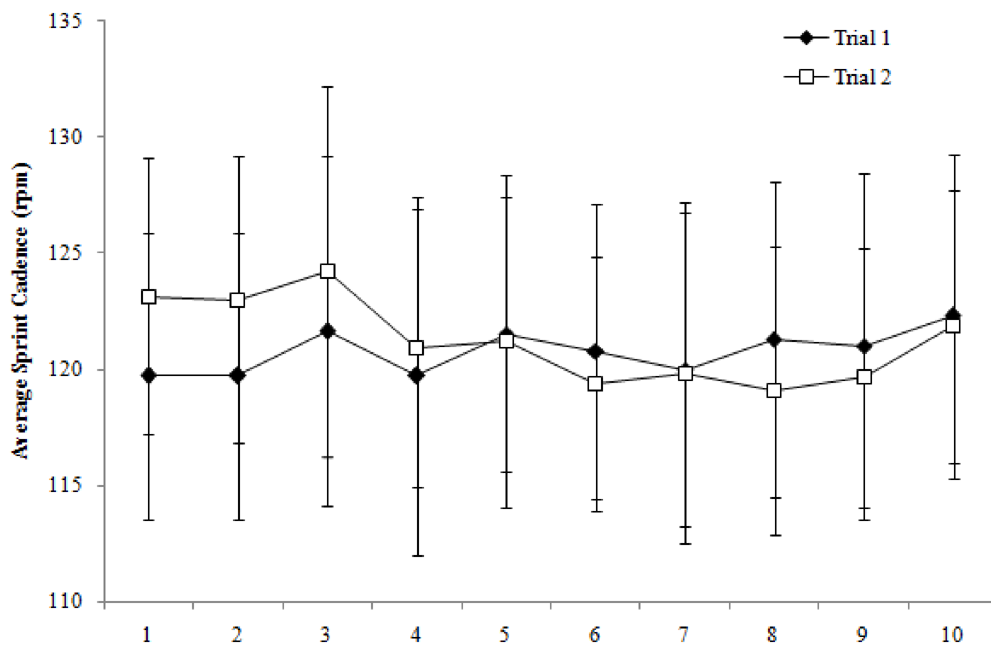

Figure 9.5 Average sprinting cadence for each sprint during both trials.

9.3.2 Hormone Concentrations

9.3.2.1 Testosterone:

A significant main effect for time-point was observed ($P = 0.001$). Total testosterone concentration was elevated above pre-exercise concentrations at all time-points ($P < 0.001$). Mean concentration increased from pre-exercise to post-20 min effort ($P < 0.01$), with further significant increases from post-20min effort to post-sprint 4 ($P < 0.01$), and from post-sprint 4 to post-exercise ($P = 0.02$).

9.3.2.2 Cortisol:

A significant main effect was observed for time-point for cortisol ($P = 0.033$). Mean cortisol concentration decreased from pre-exercise to post-20 min effort ($P < 0.05$), followed by an increase from post exercise to post-sprint 4 ($P < 0.01$) back to baseline concentrations, before a further significant increase at post-exercise ($P < 0.01$). A significant main effect was observed for trial in percentage change in cortisol concentration ($P = 0.05$). The percentage increase in cortisol concentration from post-20min effort to post-sprint 4 was smaller in the first trial in comparison to the second (2 ± 14 % *vs* 15 ±18%, $P = 0.03$) as was the increase in cortisol from pre-exercise to post-exercise (23 ± 52% *vs* 76 ± 102%, $P = 0.04$).

9.3.2.3 DHT:

A main effect for time-point was also observed for DHT ($P = 0.026$) with mean DHT concentration increasing from pre-exercise to post-20 min effort ($P < 0.05$), with a further significant increase from post-20 min effort to post-sprint 4 ($P < 0.05$). DHT concentration was elevated above pre-exercise concentrations at all time-points ($P < 0.05$).

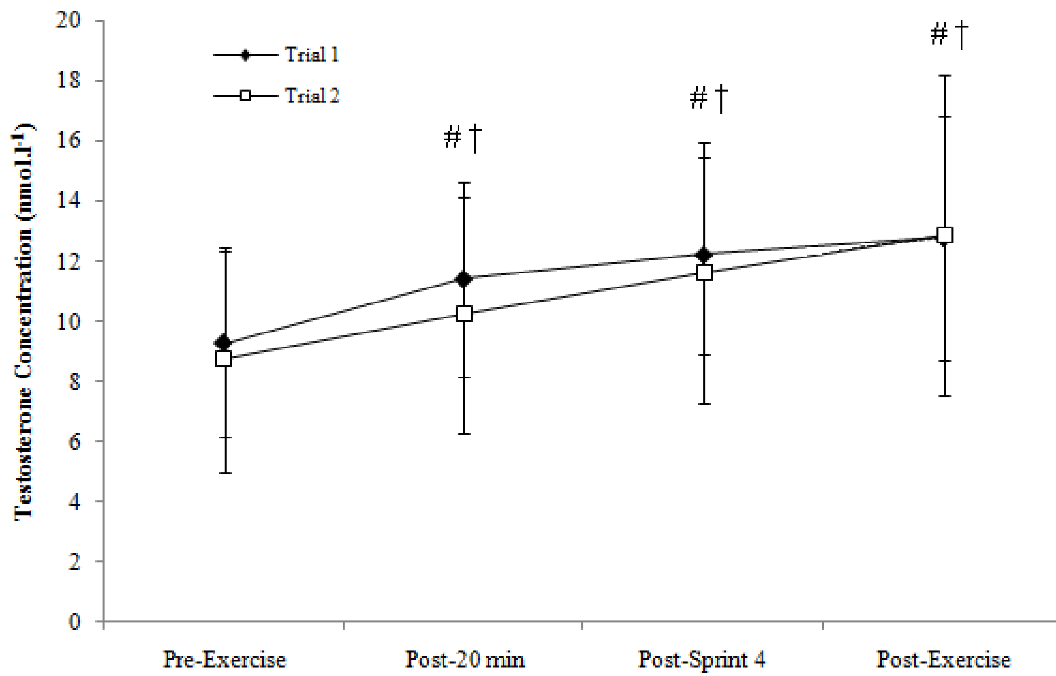

Figure 9.6 Testosterone concentration across trials. (# = Difference from pre-exercise; † = Difference from previous time-point; $P \leq 0.05$)

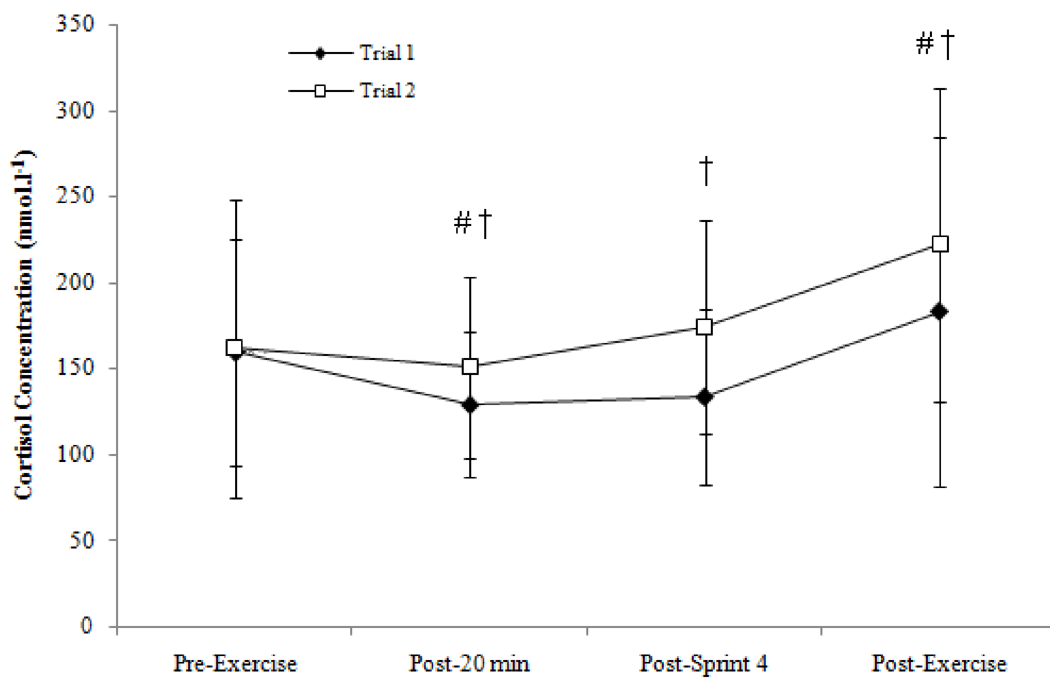

Figure 9.7 Cortisol concentration across trials. (# = Difference from pre-exercise; † = Difference from previous time-point; $P \leq 0.05$)

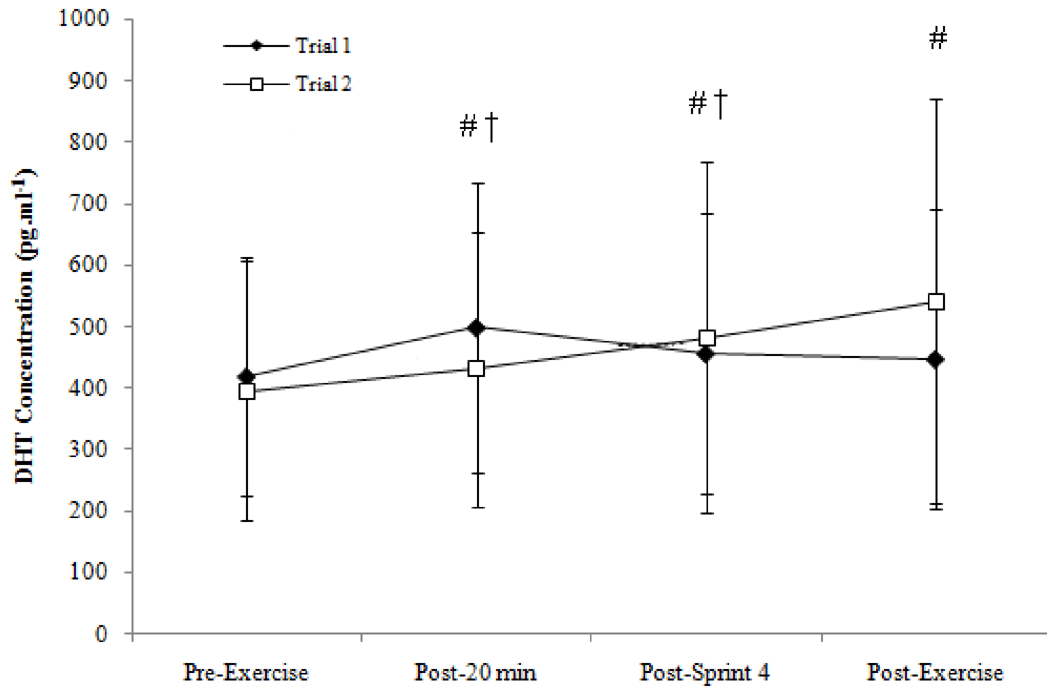

Figure 9.8 DHT concentration across trials. (# = Difference from pre-exercise; † = Difference from previous time-point; $P \leq 0.05$)

9.3.3 RPE & Psychological Affect

9.3.3.1 RPE:

No significant differences between trials were detected. A main effect was observed for time-point (P < 0.001). RPE was elevated above rest following the 20 min effort (*P* < 0.001), which further significant increases from post-20 min effort to post-sprint 4 (*P* < 0.001) and from post-sprint 4 to post-exercise (*P* < 0.01).

9.3.3.2 Psychological Affect:

A main effect was evident for time-point (*P* < 0.001) with affect was decreasing from pre-exercise to post-20 min effort (*P* < 0.001), with further significant decreases from post-20 min effort to post-sprint 4 (*P* < 0.001), and from post-sprint 4 to post-exercise (*P* < 0.001). There was a tendency for affect to be different between trials (P = 0.062). Affect was significantly lower following the 20 min effort in the second trial compared to the first (*P* < 0.05).

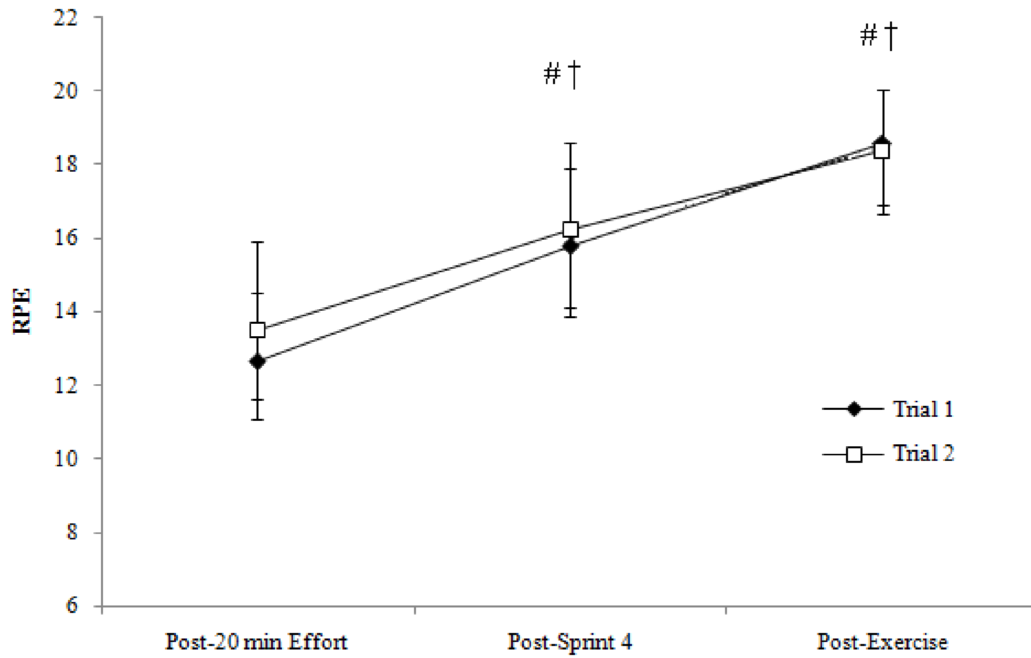

Figure 9.9 RPE across trials. (# = Difference from pre-exercise; † = Difference from previous time-point; $P \leq 0.05$)

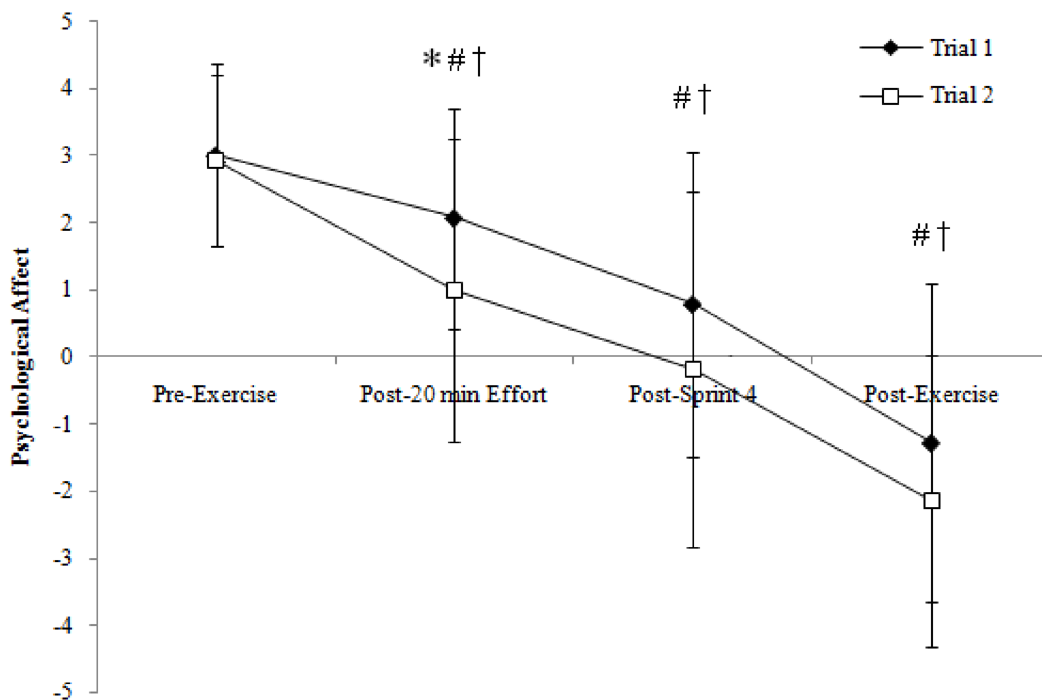

Figure 9.10 Psychological affect across trials. (* = Difference between trials; # = Difference from pre-exercise; † = Difference from previous time-point; $P \leq 0.05$)

9.3.4 Performance Parameter Correlations with Hormone Concentrations

Average power across all sprints was inversely associated with the change in cortisol concentration from post-sprint 4 to post exercise (r = -0.448; $P < 0.05$), and with post-exercise cortisol concentration (r = -0.527; $P < 0.001$). In addition, average peak power was inversely associated with percent change in cortisol concentration from post-sprint 4 to post-exercise (r = -0.432; $P < 0.01$) and post-exercise cortisol (r = -0.449; $P < 0.01$).

9.3.5 Psychological Affect and RPE Correlations with Hormone Concentrations

Post-20 min effort affect was associated with percent change in testosterone concentration from post-sprint 4 to post-exercise (r = 0.449; $P < 0.01$), and was inversely associated with post-20 min effort RPE (r = -0.674; $P < 0.001$). Post-sprint 4 affect inversely correlated with post-20 min effort cortisol concentration (r = -0.433; $P < 0.01$). Post exercise affect inversely correlated with post-20 min effort RPE (r = -0.648; $P < 0.001$) and post-exercise RPE (r = -0.527; $P < 0.001$).

Post 20-min effort RPE correlated with the change in cortisol from pre-exercise to post-20 min effort RPE (r = 0.619, $P < 0.001$). Post-sprint 4 RPE was correlated with the change in cortisol concentration from pre-exercise to post-20 min effort (r = 0.397, $P < 0.01$). Post-exercise RPE was inversely associated with pre exercise cortisol concentration (r = -0.446; $P < 0.01$) and post-20 min effort cortisol concentration (r = -0.404; $P < 0.01$).

9.4 Discussion

The present data demonstrate an increase in the concentrations of testosterone and DHT and a decrease in cortisol in response to a 20 min effort designed to elicit fatigue, with subsequent increases in concentrations of testosterone, DHT and cortisol in response to a repeated sprint cycle protocol performed immediately after. The data also reveal successive increases in RPE during trials, with simultaneous decreases in affect. Small differences in affect were observed between trials but these were not associated with changes in hormone concentrations or performance.

In terms of performance, there were no significant differences between trials in terms of average power output, cadence, or pacing during the 20 min effort, and average power was not significantly different between each 5 min period of the 20 min effort. This indicates a consistency of performance and pacing strategy by trained cyclists when asked to maintain a specific power goal, reinforcing previous findings in well-trained cyclists demonstrating reproducibility of power output and cadence across a 20 km TT (Thomas *et al.,* 2012) and 4 km TT (Stone *et al.,* 2011). For the repeated sprint bout, peak cadence for sprints 1 and 2 was significantly higher in the second trial than the first with a tendency for this to also apply to average cadence for these sprints. In addition, peak power was higher for sprint 3 in trial 2 in comparison to trial 1, with a trend for the same to apply for the second and final sprints, however, average power was not different. Although in relation to a repeated sprint protocol, these data are reflective of previous findings relating to TT performance demonstrating non-significant, greater variability in power and cadence at the beginning and end of an effort (Thomas *et al.,* 2012).

There was a tendency for affect to be different between trials at the post-20 min effort time-point, with decreased affect reported during the second trial. Further examination of the data revealed that this finding applied solely to the group of eight participants who performed their trials together. This difference may have been generated by a change in environmental conditions. Despite attempts to control laboratory conditions via air conditioning, mean ambient temperature for the second trial was significantly higher than the first trial (22.0°C *vs* 25.2°C; $P = 0.01$) with relative humidity also significantly higher (54% *vs* 59%; $P < 0.001$). Increases in ambient temperature and humidity have been shown to influence perceptions of effort (Gonzalez-Alonso *et al.,* 1999; Nielsen *et al.,* 2001; Taylor *et al.,* 2014). While RPE post-20 min effort was not significantly different between trials, it was 1.1 units higher at this time-point during the second trial and was strongly inversely correlated with affect at this time-point ($r = -0.674$; $P < 0.001$). In addition, greater negative affect, as reported at the 20 min effort in the second trial, has been linked to increased cortisol concentrations at rest and during exercise (Acevedo *et al.,* 2007), yet, despite the differences in affect, no differences were observed between trials in performance or hormone concentrations at this time-point. Taking affect as a marker of mood, this would seem to suggest that, in contrast to previous findings (Cook & Crewther, 2012a; 2012b), mood is not related to hormone response and performance in this context. Indeed, reported affect did not influence subsequent performance. During the repeated sprint protocol, peak power during was actually greater during the second trial for the third sprint with a trend for the same to apply to the second sprint and final sprints, and peak cadence for the first and second sprints was greater during the second trial, with a tendency for the same to apply to average cadence. As such, affect reported at this time point, but may merely reflect the perception of how performance of a sustained effort felt in warmer

conditions rather than a true change in mood influencing the participants motivation to execute the sprint protocol as during both trials, positive correlations were seen between affect and percentage change in testosterone, with inverse correlations observed between affect and cortisol concentration, which would lend support for previous findings (Cook & Crewther, 2012a; 2012b). In addition, warmer conditions might result in higher muscle temperature, and therefore improved sprint performance (Kilduff *et al.,* 2013). It is also likely that the attempt to influence mood in the present investigation through the use of the group setting and an unfamiliar intense protocol was not sufficient to stimulate a change in hormone concentrations. A change in affect has been achieved previously through interventions aimed at directly influencing mood through the viewing of various video clips. Aggressive, intense and positive video clips resulted in increases in endogenous testosterone and was associated with improved physical performance and skill execution (Cook & Crewther, 2012a; 2012b), while more cautionary and less positive and motivational videos were related to increased cortisol and decreased performance (Cook & Crewther, 2012a). Directly aiming to manipulate mood via a similar strategy may have induced a greater difference in affect between trial in the present investigation and also led to changes in hormone concentrations and performance.

The increase in testosterone in response to the 20 min intense pre-fatigue effort is in line with previous observations demonstrating an increase in testosterone in response to a 65 min effort at 75% VO_2max (Ronsen *et al.,* 2001). The increase seen in concentrations of testosterone, DHT and cortisol in relation to the repeated sprint protocol are also in agreement with those we have observed previously following the same protocol (Chapter 7 & 8; Smith *et al.,* 2013). There were no differences

between trials in concentrations of testosterone, DHT and cortisol at any time-point. However, there were differences between trials in terms of percentage change. The greater percentage increase in testosterone and cortisol concentrations from post-20min effort to post-sprint 4 and in cortisol concentrations from pre-exercise to post-exercise during the second trial in comparison to the first may be related to the aforementioned differences in cadence and power across the first three sprints in the second trial. Increased cadence has previously been shown to be related to greater elevations in androgens (Smith *et al.,* 2013), however percentage change in DHT was not different between trials. Alternatively, it could be suggested that the greater metabolic stress, elicited by via increased peak power and cadence, contributed to this difference, with an increase in metabolic stress having been previously linked to an increase in testosterone response (Goto *et al.,* 2005). In addition, and as previously mentioned, ambient temperature was higher during the second trial for one of the groups, and warmer temperatures have also been shown to result in greater increases in concentrations of cortisol and testosterone as a result of the greater overall stress (Maresh *et al.,* 2014).

We have previously shown increases in cortisol and testosterone in response to the repeated sprint bout when performed alone to be an average of 17% for testosterone and 74% for cortisol (Chapter 7). Present changes in concentrations of testosterone and cortisol from pre-exercise to post-exercise following both the 20 min effort and the repeated sprint protocol were 45 ± 28% and 48 ± 83% respectively. While caution should be employed to comparing between studies given the individual variation in responses seen, these data would suggest that the pre-fatiguing bout did not blunt HPA axis sensitivity and the hormone response to the subsequent repeated sprint bout.

Conversely, it may have enhanced the testosterone response while subduing the cortisol response. In addition, and as previously mentioned, metabolic stress has been linked to the magnitude of testosterone response during resistance exercise (Goto *et al.,* 2005). It is likely the metabolic stress induced by the 20 min effort would have possibly contributed to enhancing the testosterone response to the repeated sprint bout.

Interestingly, peak power and average power across all sprints were inversely associated with the increase in cortisol concentration from post-sprint 4 to post exercise, and with post-exercise cortisol concentration. This may indicate a relationship between cortisol response and training status. Higher peak power and average power across the repeated sprint protocol would be associated with more highly trained individuals, suggesting that the response of cortisol to a repeat sprint cycling protocol is decreased in these individuals. While all participants in the present study were trained, this relationship may reflect the extent of this training status. Cortisol response has previously been associated with training status (McMillan *et al.,* 1993; Crewther *et al.,* 2006) and was also evident in our previous research on the response to the repeated sprint bout in women (Chapter 8).

9.5 Conclusion

In conclusion, the present data demonstrate an increase in the concentrations of testosterone and DHT and a decrease in cortisol in response to a 20 min effort designed to elicit fatigue, with subsequent increases in concentrations of testosterone, DHT and cortisol in response to a repeated sprint cycle protocol performed immediately after. The data suggest no influence of the pre-fatigue effort on the ability of the endocrine system to respond subsequent exercise. Successive increases in RPE were observed during trials, with simultaneous decreases in affect. Small difference in affect between trials were noted following the 20 min effort, but were not associated with differences in hormone concentrations and did not influence performance and are likely to have been related to differences in environmental conditions.

- Chapter 10 -

Acute Hormonal and Neuromuscular Response to Priming Exercise and its Influence on Performance

10.1 Introduction

Circulating concentrations of androgens, in particular testosterone, have been linked to enhanced performance in strength and power tasks (Cardinale & Stone, 2006; Crewther *et al.,* 2012a; Crewther *et al.,* 2012b) and correlated with neuromuscular performance (Crewther *et al.,* 2009). One of the mechanisms by which steroid hormones may achieve this is by influencing neuromuscular function (Crewther *et al.*, 2011a). The short-term effects of steroid hormones on the neuromuscular system include facilitation of rapid activation of many second messenger signals such as the intracellular release of ions such as ATP, calcium and potassium in different cells (Passaquin *et al.,* 1998; Jaimovich & Espinosa, 2004; Han *et al.,* 2005), with various downstream effects on protein kinase pathways (Estrada *et al.,* 2003; Han *et al.,* 2005; Nguyen *et al.,* 2005). Calcium release into skeletal muscle cells is therefore influenced by testosterone and cortisol concentrations, with calcium subsequently involved in twitch relaxation, energy metabolism and the structural integrity of the muscle fibre in muscle fibre (Berchtold *et al.*, 2000). These signalling effects can influence neuronal excitement within minutes (Smith *et al.,* 2002; Zaki & Barrett-Jolley, 2002). Therefore, the hormonal stimulation of these signals could influence the functioning of the neuromuscular tissue and system.

Steroid hormones can also influence the action of the motor system. Low free cortisol has been linked to enhanced motor cortex response (Sale *et al.,* 2008), while

exogenous testosterone has decreased cortical motor threshold in humans (Bonifazi *et al.,* 2004), and has been linked to improved performance via the influence on skeletal muscle function. Recently, the concept of hormonal priming has received attention. This concept relates to a potential association between concentrations of endogenous testosterone and elements of athletic behaviour linked to confidence and motivation to compete, and may result from the effects of endogenous testosterone on the brain (Cook & Crewther, 2012a). This theory has been supported through observations that elevations in total testosterone and free testosterone concentration prior to performance, through the viewing of various video clips, enhanced motivational behaviour and performance of identified skills (Cook & Crewther, 2012b), as well as improved 3RM back squat performance (Cook and Crewther, 2012a).

The process of postactivation potentiation (PAP) has been shown to enhance muscular performance in endurance activities involving speed and power (Hodgson *et al.,* 2005). Mechanisms via which PAP is suggested to exert its effect include phosphorylation of myosin regulatory light chains making actin and myosin more sensitive to calcium, an increase in α-motoneuron excitability as reflected by the changes in the H-reflex, and changes in pennation angle of the muscle influencing force transmission (Tillin & Bishop 2009). Different conditioning protocols have been utilised to induce PAP. Multiple sets of the conditioning stimulus have been demonstrated to be more effective than a single set, and high intensity conditioning stimuli have been shown to enhance performance to a greater extent than moderate or low intensity conditioning activity (Wilson *et al.*, 2013). The mode of conditioning stimulus used most frequently within the literature has been leg press or squat movement performed at a high percentage of 1 RM, with the efficacy of the stimulus

assessed using jump performance. However, different modes of pre-exercise conditioning activities have been shown to enhance performance of explosive activities, in particular the use of single cycle sprints, with the potentiating effect of the cycle sprint associated with elevations in salivary testosterone (Crewther *et al.,* 2011b). In support of these observations, more recent research has demonstrated further support for a potentiating effect of low-load ballistic exercise on performance of strength and power task such as jumps and sprints (Maloney *et al.,* 2014). Ballistic exercise is defined by performing movement with maximal velocity, involving mass being accelerated throughout an entire movement. This removes the braking phase associated with heavy resistance exercise, facilitating greater muscle activation and force output (Newton *et al.,* 1996). It is thought that ballistic exercise is able to induce post-activation potentiation via high recruitment of type II muscle fibres, that are associated with its performance, as well as through augmenting lower limb stiffness.

The aim of the present investigation was: i) to assess whether different methods of priming or potentiating exercise would be successful in enhancing power and strength performance, and ii) if potentiation occurred, to assess whether it was associated with elevations in hormone concentrations and muscular activation.

10.2 Method

10.2.1 Participants

Eleven young, healthy males (age: 24.8 ± 3.7 y; body mass 77.3 ± 7.5 kg; leg press 1 RM 325 ± 63 kg) participated in this study. All participants completed resistance training 3 - 5 times each week, and had a resistance training history of a minimum of two years. Each participant was briefed regarding the nature of the study and provided written informed consent prior to commencing any testing. This study was approved by the University of Bath Research Ethics Committee.

10.2.2 Preliminary Measurements

Preliminary tests were conducted in order to determine each participant's one repetition maximum (1RM) on leg press, maximal vertical jump height, and maximal power during a 10 s cycling sprint test. Participants performed a warm up at a self selected in intensity on a stationary cycle ergometer (Wattbike Ltd, Nottingham, UK), before performing 10 s maximal sprint from a standing start. Following this, participants performed a maximal countermovement jump using a displacement transducer (GymAware, Kinetic Performance Ltd, Mitchell, ACT, Australia). The transducer consists of a linear encoder unit that relays information, via infrared connection, to a hand held device. The time-displacement data are used to calculate movement velocities and subsequent accelerations. Data are then differentiated, and the kinematic data used to estimate force and power when external load and/or body mass are included (Crewther *et al.,* 2011). For force and power measurement, CVs of 2.5% and 3.0% respectively, have previously been reported with use of the GymAware system (Crewther *et al.,* 2011d), along with significant correlations to force plate data (Cronin *et al.,* 2004; Crewther *et al.,* 2011d). Finally, participants

completed an incremental strength test to determine seated leg press 1RM (Keiser UK, Tetbury, UK). Data were used in order to establish maximal performance data for each participant and to calculate workload for subsequent trials. Participants were asked to refrain from strenuous exercise and to avoid both alcohol and caffeine consumption during the 24 h prior to the main trials.

10.2.3 Experimental Design

Participants completed four main trials in a randomised counterbalanced order, separated by a minimum of 48 h. All trials consisted of a standardised 5 min warm up relative to body mass on the stationary cycle ergometer before completion of one of three different priming exercise bouts, or a control. The three different priming bouts were i) 3 x 1 repetition of heavy leg press at 90% of 1 RM with 50 s between repetitions (HLP); ii) 3 x 10 s high cadence sprints on the stationary cycle ergometer at moderate resistance, a cadence of 130-140 rpm and 50-60% peak power with 50 s between repetitions (HCS); iii) 3 x 10 s low cadence sprints on the stationary cycle ergometer at high resistance, a cadence of 50-60 rpm and 50-60% peak power with 50 s between repetitions (LCS). The control trial (CTL) consisted of completing the same 5 min warm up, before being seated. Following the priming exercise bouts participants were asked to take a seat for 8 min before performance of a maximal vertical jump, immediately followed by a set to fatigue of leg press (LPF) at 80% of 1RM. Trials were performed while wearing a pair of fitted shorts with integrated electromyography (EMG) sensors at the quadriceps, hamstrings and gluteals (Myontec Ltd, Kuopio, Finland). EMG signal was transmitted via bluetooth to a laptop.

10.2.4 Sampling and Analysis

Blood Sampling: All trials were performed in the morning, starting between 8am and 11am. Each participant arrived at the laboratory at the same time of day for each trial, non-fasted. A pre-exercise blood sample was obtained from a finger tip into serum collection tubes (Serum Z/500 ul; Sarstedt, Germany). Further blood samples were obtained post-stimulus, mid-way through the 8 min recovery period between the priming exercise or control and the performance tests, and post-exercise following the conclusion of the set of leg press to fatigue. Samples were left to clot for 15 min at room temperature before being centrifuged at 3000 rpm (1500 g) for 10 min. Serum was then transferred to labelled Eppendorfs and frozen at -20°C until further analysis. Samples were analysed in duplicate via ELISA for total testosterone, free testosterone, DHT and cortisol using commercially available kits (IBL, Hamburg, Germany). Combined intra- and inter-assay variation was 4.6% and 5.7% respectively for total testosterone, 4.1% and 5.4% for free testosterone, 6.9% and 8.5% for DHT, and 3.0% and 3.5% for cortisol.

EMG: EMG signal was recorded throughout each trial at 1,024 Hz. All data were filtered with a band-pass filter allowing 400 Hz high pass and 10 Hz low pass with use of the MyOnWear software (Myontec Ltd, Kuopio, Finland). Filtered files were exported for analysis in Microsoft Excel (version 2007). Raw signal was full-wave rectified and smoothed over 50 ms. Average rectified EMG (AREMG) and peak EMG (PEMG) were calculated from the rectified signal across the first five repetitions of the set of LPF and for the vertical jump.

10.2.5 Statistical Analyses

Two-way repeated measures ANOVA was used to investigate differences between trial and time-point for all measured hormones and for performance parameters and EMG signal in the first five reps of the set of leg press to fatigue. One way-repeated measures ANOVA was used to investigate differences in jump performance, number of repetitions during LPF, and percentage change in hormones between trials. Subsequent two-tailed paired T-tests, with post hoc Bonferroni correction, were carried out to determine the location of any variance. Pearson product moment correlations were performed to assess relationships between variables. All analyses were conducted using IBM SPSS Statistics (version 21.0.0; IBM, New York, NY). All data are presented as mean ± SD. Significance was accepted at $P \leq 0.05$.

10.3 Results

10.3.1 Performance Parameters

Vertical jump height was not significantly different between trials (Fig. 10.1), however there was a tendency for jump peak power to be different between trials (P = 0.07) (Fig. 10.2). Number of repetitions during the set of leg press to fatigue was not different between trials (Fig. 10.3) and no significant difference was apparent between trials in peak power across the first five repetitions of the set of leg press to fatigue, however, there was a significant main effect for repetition for peak power (P = 0.005) (Fig. 10.4). Further investigation revealed power for repetition 1 to be significantly lower than repetitions 2, 3, 4 and 5 ($P < 0.01$).

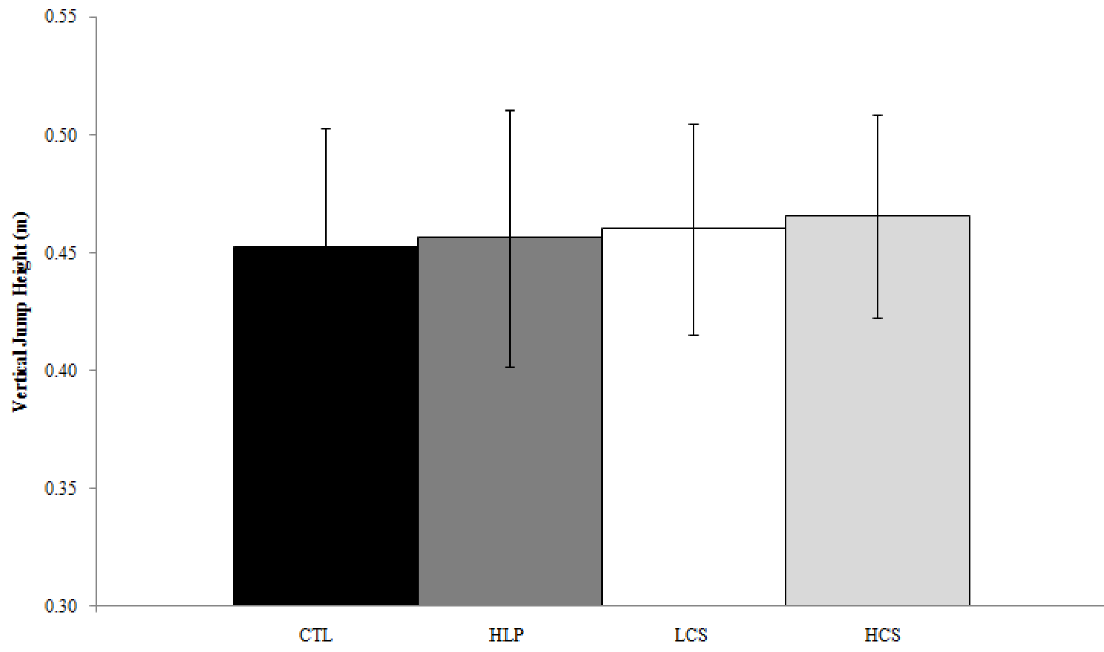

Figure 10.1 Mean countermovement jump height attained in each trial.

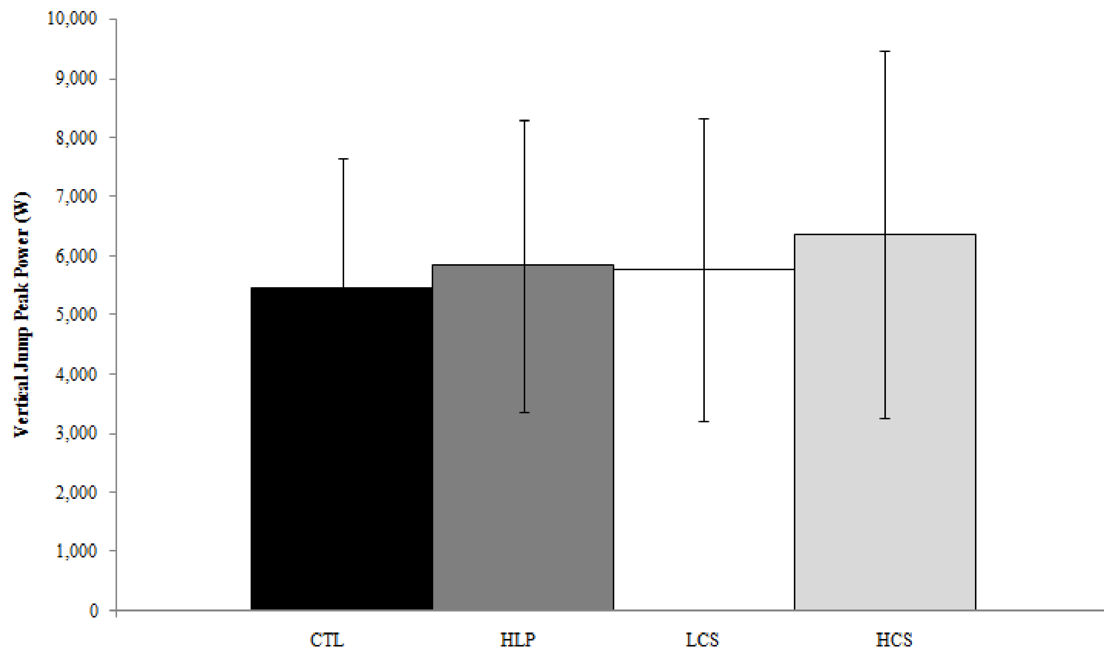

Figure 10.2 Mean vertical jump peak power attained in each trial.

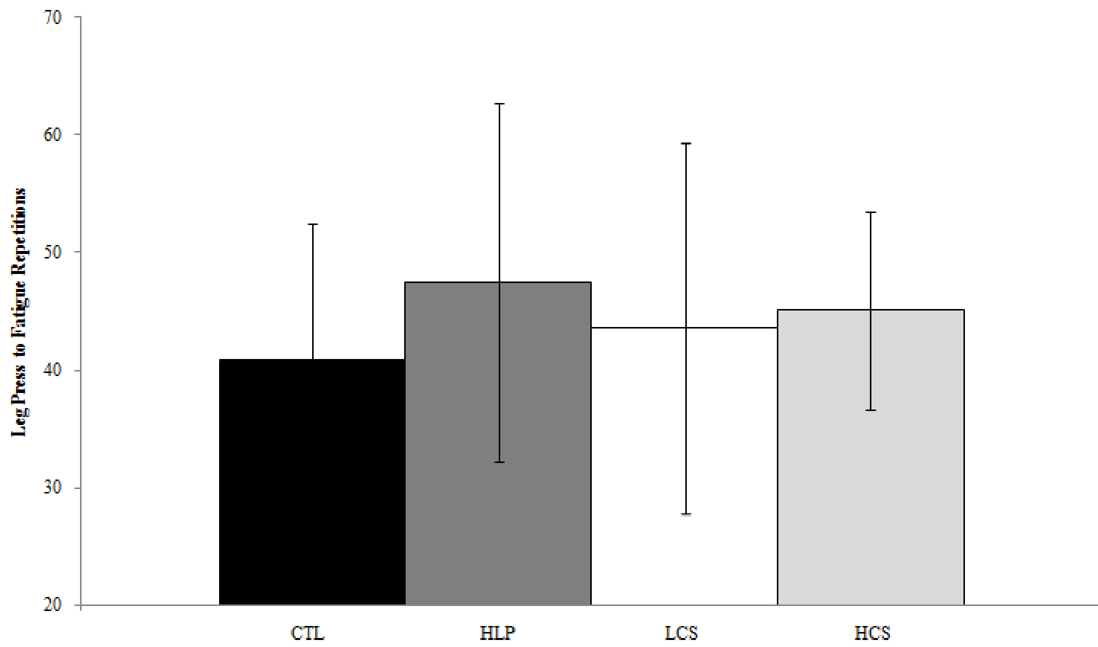

Figure 10.3 Mean number of repetitions completed during leg press to fatigue in each trial.

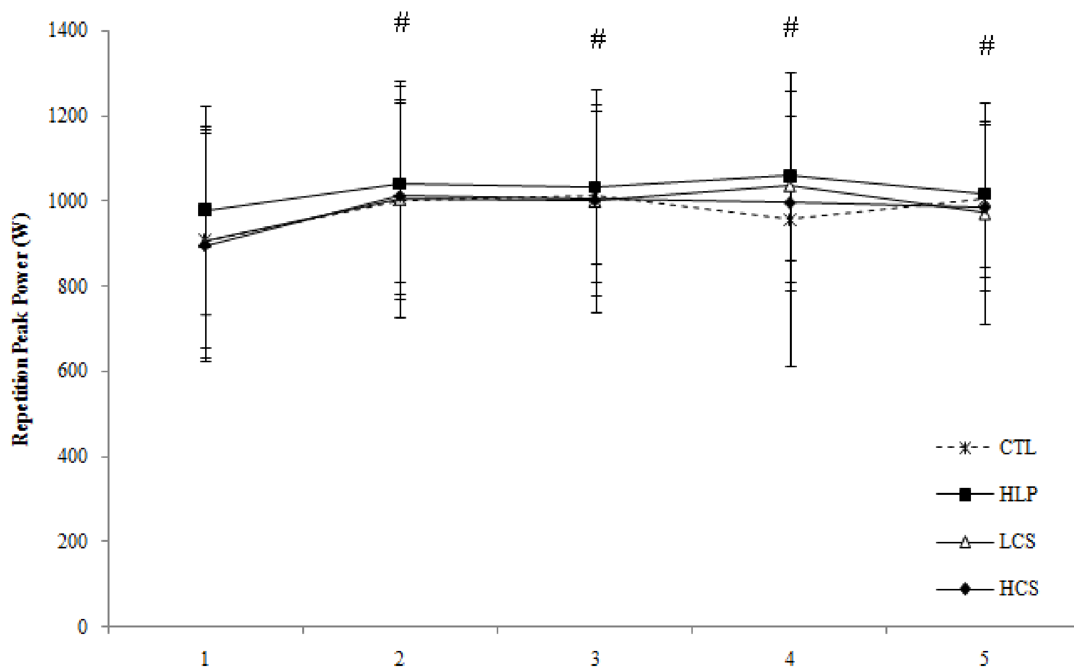

Figure 10.4 Mean peak power of each of the first five repetitions during leg press to fatigue for each trial (# = Difference from repetition 1; $P < 0.05$)

10.3.2 Hormone Concentrations

10.3.2.1 Total Testosterone

Total testosterone concentration was not significantly different between trials at any time-point, however, a main effect for time was apparent ($P < 0.001$) (Fig. 10.5). In all trials, total testosterone concentration increased from pre-exercise to post-exercise ($P < 0.05$). In all trials, except for HCS, total testosterone concentration increased significantly from post-stimulus to post-exercise ($P < 0.05$). Percentage increase in total testosterone from pre- to post-exercise was significantly greater in the HCS trial in comparison to CTL (12 ± 11% *vs* 23 ± 10%; $P = 0.03$).

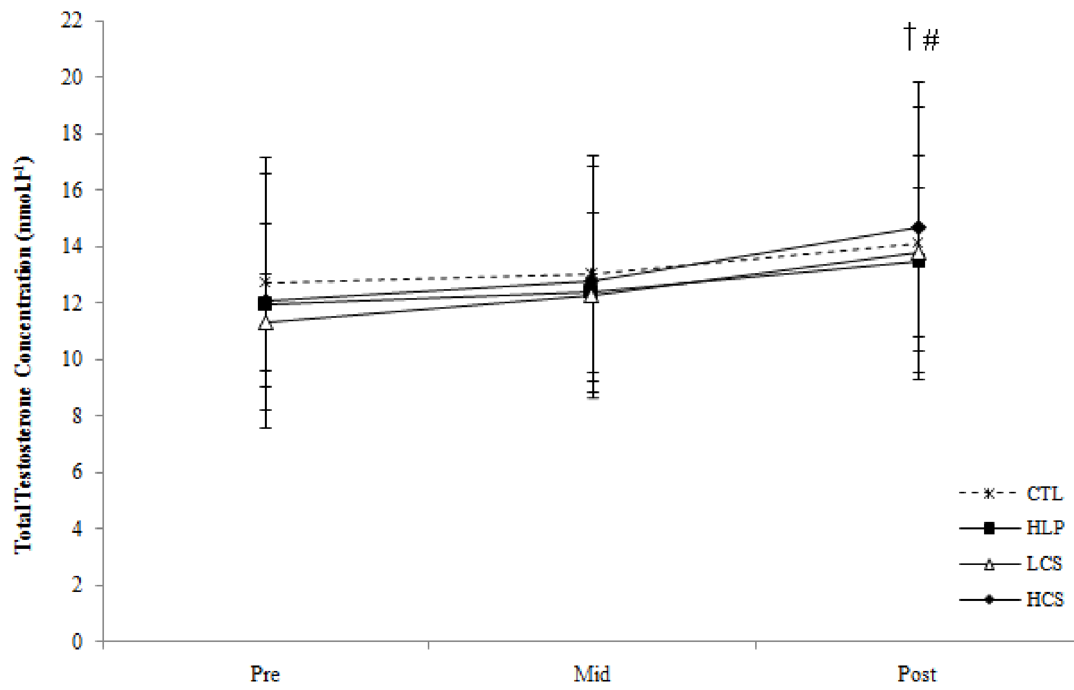

Figure 10.5 Mean total testosterone concentration over the course of each trial. (# = Difference from pre-exercise; † = Difference from mid-recovery period; $P < 0.05$)

10.3.2.2 DHT

DHT concentration displayed a significant main effect for time (P = 0.03), with a tendency for there to be an interaction effect between time-point and trial (P = 0.07) (Fig. 10.6). DHT concentration increased significantly from pre- to post-exercise in all trials (P < 0.05), with a significant increase in DHT concentration from pre-exercise to post-stimulus in the HCS trial (P < 0.001), and from post-stimulus to post-exercise in HCS (P < 0.005) and HLP trials (P < 0.05), with a tendency for the same to apply in the LCS trial (P = 0.07).

There was also a significant difference between trials in terms of percentage change in DHT concentration from pre- to post-exercise (P = 0.04). Percentage change in DHT concentration from pre-exercise to post-stimulus was significantly greater during the HCS trial (10 ± 7%) than during the CTL trial (3 ± 7%; P = 0.03), the HLP trial (2 ± 7%; P = 0.03), with a tendency for the increase to be greater compared to the LCS trial (4 ± 8%; P = 0.07). Percentage increase in DHT concentration from pre- to post-exercise was significantly greater during the HCS trial (19 ± 10%) than during the CTL trial (7 ± 8%; P = 0.02), and the LCS trial (10 ± 11%; P = 0.02), and a tendency for the same to apply in the HLP trial (9 ± 13%; P = 0.07).

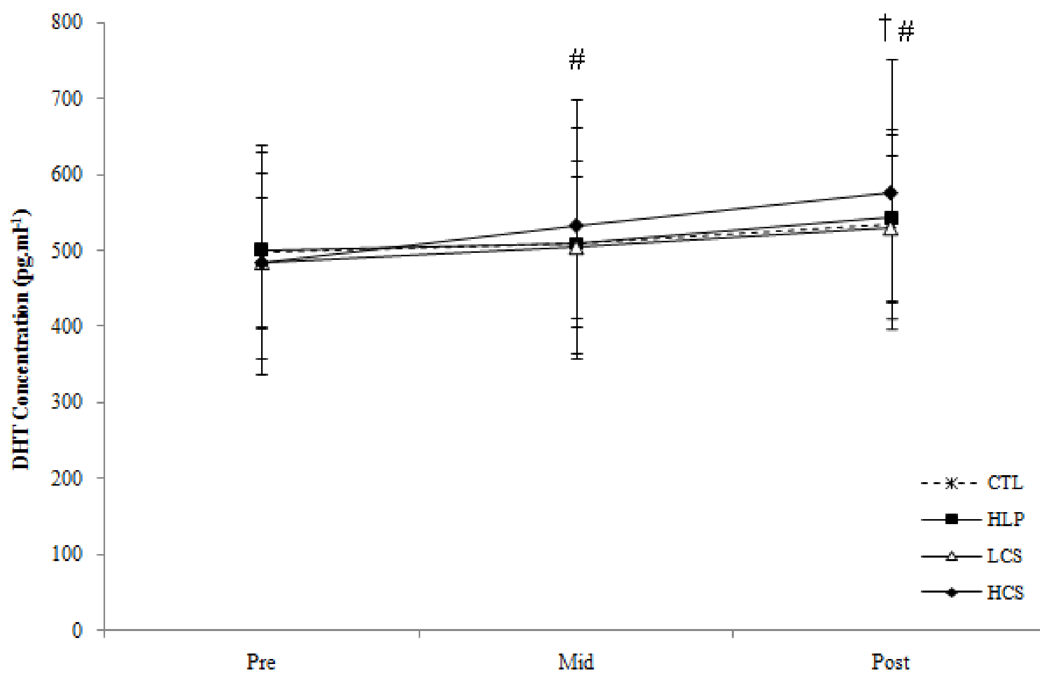

Figure 10.6 Mean DHT concentration over the course of each trial. (# = Difference from pre-exercise; † = Difference from mid-recovery period; $P < 0.05$)

10.3.2.3 Free Testosterone

Free testosterone was not significantly different between trials at any time-point, however there was a main effect for time ($P < 0.001$) (Fig. 10.7). Free testosterone concentration increased from pre- to post-exercise in all trials ($P < 0.05$), with a significant increase from pre-exercise to post-stimulus in the HCS trial ($P < 0.005$). There was a significant difference between trials in percentage change in free testosterone concentration from pre-exercise to post-stimulus ($P = 0.01$), with a tendency for there to be a difference between trials in change in free testosterone concentration from post-stimulus to post-exercise ($P = 0.06$).

Further analysis revealed percentage increase in concentration from pre-exercise to post-stimulus to be greater in the HCS trial (34 ± 25%) than in the HLP (6 ± 18%; $P = 0.01$), and LCS (6 ± 22%; $P = 0.01$) trials with a trend for the same to apply during the CTL trial (16 ± 20%; $P = 0.06$). Percentage change from post-stimulus to post-exercise was less in the HCS trial (6 ± 11%) than during the HLP trial (23 ± 15%; $P = 0.006$), and percentage change from pre- to post exercise was greater during HCS trial (42 ± 24%) than the CTL trial (27 ± 20%; $P = 0.05$).

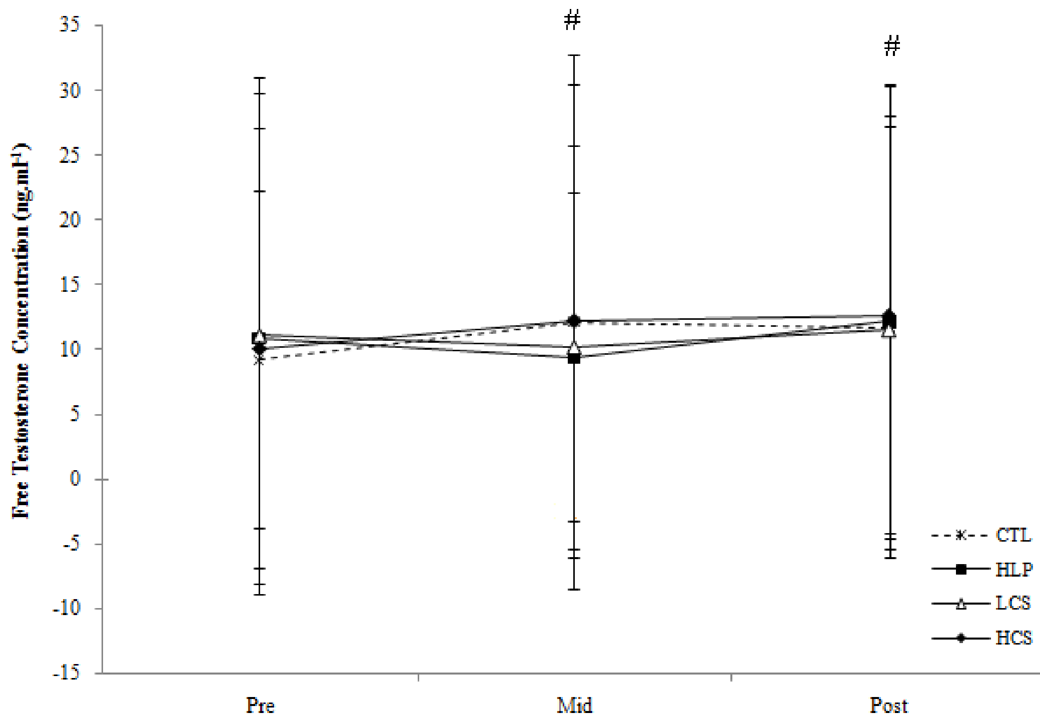

Figure 10.7 Mean free testosterone concentration over the course of each trial. (# = Difference from pre-exercise; † = Difference from mid-recovery period; $P < 0.05$)

10.3.2.4 Cortisol

Cortisol concentration displayed a significant main effect for time (P = 0.04), but was not different between trials at any time-point (Fig. 10.8). Further analysis demonstrated a significant decrease from pre-exercise to post-stimulus during the HLP trial (P = 0.002), with a tendency for the same to apply during the LCS trial (P = 0.06). In the CTL trial there was also a significant decrease in concentration from pre- to post-exercise (P = 0.02).

There was a significant difference between trials in percentage change in cortisol concentration from post-stimulus to post-exercise (*P* = 0.04). Percentage decrease from pre- to post-exercise during the CTL trial (-11 ± 11%) was greater than that during the HCS trial (-1 ± 14%; P = 0.03), with a tendency for the decrease from post-stimulus to post-exercise (-5 ± 10%) and pre- to post-exercise (-15 ± 13%) to be greater than the HCS trial (16 ± 37%; P = 0.08 and -1 ± 14%; P = 0.08, respectively).

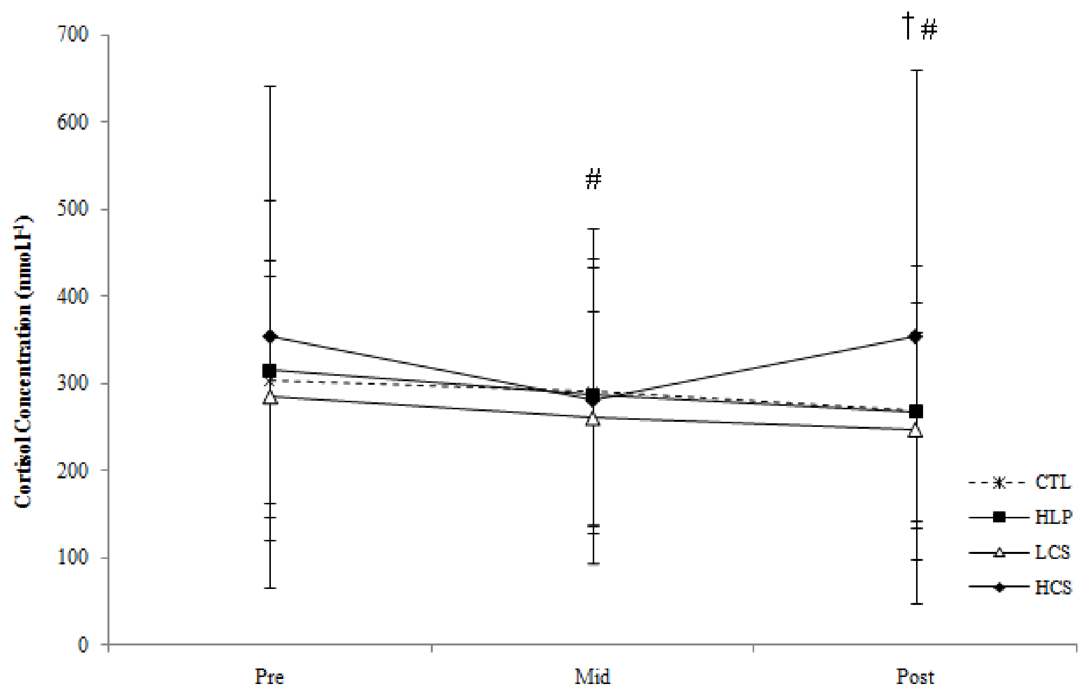

Figure 10.8 Cortisol concentration over the course of each trial. (# = Difference from pre-exercise; † = Difference from mid-recovery period; $P < 0.05$)

10.3.3 EMG Signal

Quadriceps AREMG during vertical jump was significantly different between trials ($P = 0.041$) (Fig. 10.9), with a trend for the same to apply to quadriceps PEMG ($P = 0.06$) (Fig. 10.10). Further analysis revealed quadriceps AREMG to be greater during LCS trial than CTL ($P = 0.03$), with a tendency for it to be greater during HCS trial than CTL ($P = 0.08$) and HLP trial ($P = 0.06$). There was a trend for quadriceps PEMG to be greater during LCS trial than CTL ($P = 0.06$) and greater during the HCS trial than the HLP trial ($P = 0.07$).

A significant main effect for repetition was apparent in quadriceps AREMG in the first five repetitions of LPF ($P = 0.05$), as it was for quadriceps PEMG ($P = 0.005$) however, neither was different between trials. Further analysis demonstrated quadriceps AREMG to be lower on repetition 1 than on repetitions 2 - 5 ($P < 0.02$), and quadriceps PEMG was also significantly lower on repetition 1 than on repetitions 2 - 4 ($P < 0.02$), with PEMG being significantly lower for repetition 5 than for repetition 2 ($P = 0.02$) and repetition 3 ($P = 0.03$).

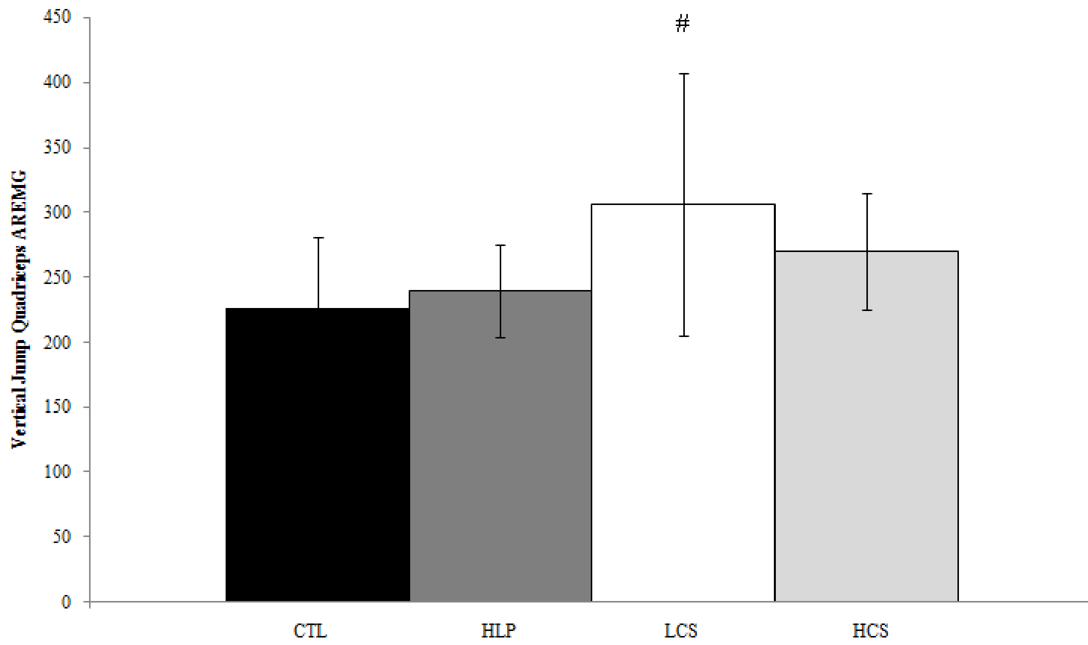

Figure 10.9 Quadriceps AREMG during vertical jump for all trials (# = Difference from CTL trial; $P < 0.05$)

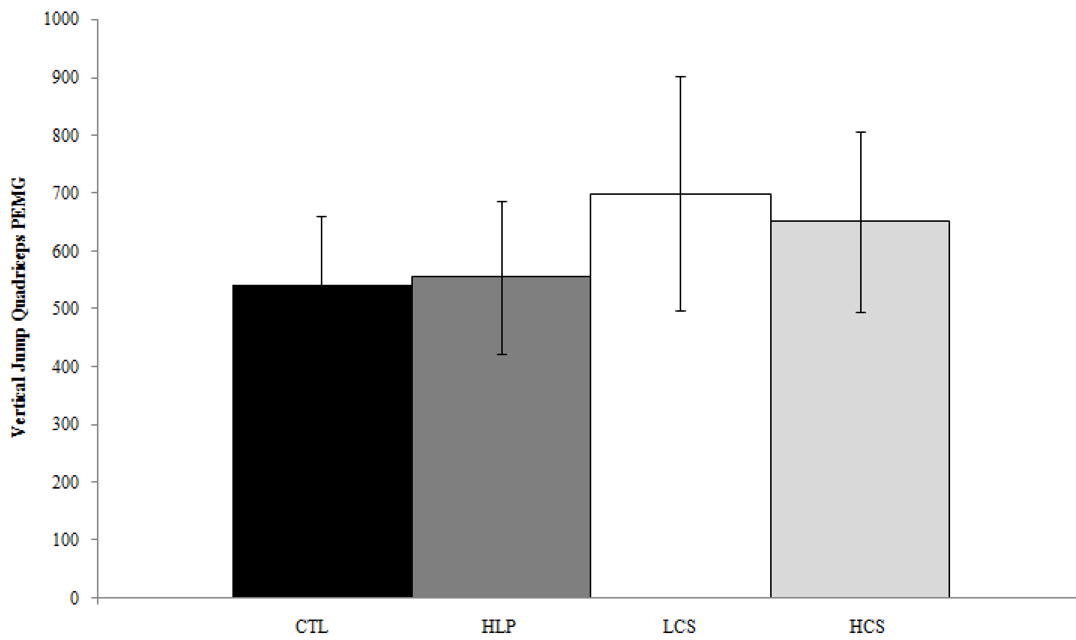

Figure 10.10 Quadriceps PEMG during vertical jump for all trials.

10.4 Discussion

The present data do not demonstrate an enhancement of vertical jump performance or number of repetitions during performance of leg press exercise to fatigue with any of the potentiating exercises used in this investigation, suggesting postactivation potentiation did not occur. Given this observation, the secondary aim of the study, to assess the association between hormone concentrations and potentiation, was disregarded. However differences were observed between trials in muscular activation of the quadriceps during vertical jump performance.

To our knowledge, this is the first research to investigate EMG activity alongside concentrations of serum hormones and exercise designed to potentiate subsequent muscle function. However, as stated, no potentiating effect was evident. Previous studies have also shown absence of performance improvement in vertical jump height following potentiating stimuli (Chiu *et al.,* 2003; Khamoui *et al.,* 2009). Where participants have been recreationally trained, postactivation potentiation has been shown to be less effective (Chiu *et al.,* 2003; Khamoui *et al.,* 2009; Wilson *et al.,* 2013). In a meta-analysis, effect sizes for muscle power following postactivation potentiation were reported as 0.14, 0.29 and 0.81 for untrained individuals, trained individuals and athletes, respectively (Wilson *et al.,* 2013). While the participants in the current investigation were all trained, and regularly completed resistance training there was large variation in 1 RM, as well as the sports for which participants trained. This generates a large amount of variation in comparison to studies that have shown strong effects when using squads of elite athletes completing the same training (Crewther *et al.,* 2009, 2011b, 2011c). Following potentiation, optimal performance occurs when fatigue has subsided, but the potentiated effect still exists (Hodgson *et*

al., 2005). Thus, inherently, training status has been linked to most effective duration of the recovery period, with trained individuals seemingly responding better to a longer recovery period than athletes (Wilson *et al.,* 2013). While a number of recovery periods of different duration have been investigated, the most beneficial in enhancing performance of a countermovement jump has been shown to be approximately 8 min (Kilduff *et al.,* 2007; Kilduff *et al.,* 2008; Wilson *et al.,* 2013) However, it is possible that participants in the present investigation may have required a longer recovery period in order for the potentiating exercises to be effective.

The effectiveness of postactivation potentiation can also be influenced by muscle fibre type distribution. Individuals with a higher proportion of type II fibres have previously demonstrated a greater postactivation potentiation response but also generated a higher fatigue response (Hamada *et al.,* 2003). This raises the possibility that for optimal performance, postactivation potentiation protocols should be individualised. Indeed, the effectiveness of postactivation potentiation in athletes has been shown to be enhanced by individualising the recovery period to each athlete (Crewther *et al.,* 2011c). Participants in the present investigation would fall at a different points on a continuum of each of both training status and fibre type distribution, introducing variability into the observed data, as can be seen by the magnitudes of the standard deviations, and may also explain the lack of postactivation potentiation response seen.

Previous research has considered the relationship between salivary testosterone and cortisol concentrations and performance of squat and sprint activities (Crewther *et al.,* 2009; 2011b) and demonstrated elevations of both hormones following cycle sprint

activities that was associated with improved jump performance (Crewther *et al.,* 2011b). Although total testosterone concentration in the present investigation did increase in response to all potentiating activities, it did not reach significance. However, a significant increase in free testosterone and DHT concentrations from pre-exercise to post-stimulus was observed during the HCS trial, but not during the LCS trial. This is in agreement with our previous data demonstrating increase in DHT in response to repeated sprint cycling with the magnitude of increase being related to cadence (Smith *et al.,* 2013). DHT has recently received attention for its potential to enhance performance above that of testosterone (Bauer *et al.,* 2000; Grino *et al.,* 1990). Administration of DHT to muscle fibre bundles has been shown to enhance contractile function, but only in fast-twitch fibres (Hamdi & Mutungi, 2010). As such, the presence of elevated DHT may be of considerable importance for optimal performance of high intensity strength and power based tasks. In the present investigation, although not significant, vertical jump height, and particularly vertical jump peak power, were greater during HCS trial than any other trial.

In terms of muscle activation, quadriceps AREMG during vertical jump performance was greater during the LCS trial than CTL, with a tendency for AREMG to be higher during the HCS trial than both HLP and CTL trials. Allied with this, there was a trend for PEMG to also be greater during LCS than CTL trial and during the HCS than HLP trial. These data may be indicative of increased muscular recruitment in response to both the low cadence and high cadence stimuli, above that of heavy leg press stimuli and the control. Although, as stated, no significant difference in vertical jump height was observed between trials, these EMG data are reflective of the trends observed for vertical jump performance where for eight of the eleven participants, greatest jump

height was attained during HCS or LCS trials. These data may tentatively suggest that, although not statistically significant, there was a trend for both HCS and LCS to enhance muscular activation and recruitment, and generate an improvement in the performance of an explosive power task. Considered alongside the free testosterone and DHT responses during the HCS trial, where significant increases were seen in response to the potentiating stimulus, a further tentative suggestion could be made of a link between androgen response and explosive power performance. Indeed, although differences are small and statistically non-significant, vertical jump height was on average greatest during the HCS trial. It is possible that the use of a linear displacement transducer for assessment of jump height is not sufficiently sensitive or reliable. Although validity and reliability has been established for estimates of force and power using this method (Cronin *et al.,* 2004; Crewther *et al.,* 2011), total displacement during a jump is likely to be more variable due to the possibility of the participant moving away from a true vertical during the jump, and thus introducing variability in jump height to different degrees depending on the extent of movement. In addition, as previously mentioned, the variation in the study resulting from inter-individual differences in training status and muscle fibre type distribution may also have influenced the EMG data. All participants in the current investigation wore the same pair of shorts for measurement of EMG. While this allows for a degree of intra-individual consistency in terms of sensor placement, the anthropometry of the individuals was very different. As such, the sensors would have been located in different positions on the muscle for different individuals, introducing additional variation. However, small effects and trends as noted above were still observed. It is possible that with a more homogenous and highly trained group of individuals these trends may have become more apparent.

No difference was seen between trials for quadriceps AREMG or PEMG for the first five repetitions during LPF. However, AREMG was lower on the first repetition than on the following four, with PEMG also lower on the first repetition than on repetitions two to four, and lower on repetition five than on repetitions two to three. The data also demonstrate that, following reaching maximal values at repetition two or three, PEMG began to decline by repetition five although AREMG still remained elevated at this point. These observations are likely to be linked to the muscular recruitment strategies used to generate force throughout the set of leg press exercise, but further analysis would be required in order to determine this. However, no difference was seen between trials in terms of this change in PEMG and AREMG, suggesting no influence of any of the potential potentiating stimuli on muscular activation during the performance of leg press exercise.

10.5 Conclusion

In conclusion, the present data do not demonstrate an enhancement of vertical jump performance or in number of repetitions performed during leg press exercise to fatigue with any of the exercises designed to induce potentiation in this investigation, suggesting PAP did not occur. As such, no association was established between potentiation and the hormone response to the stimuli utilised. Differences in muscular activation during jump performance were observed suggesting that low and high cadence sprints generated increased muscle recruitment during this task, however, this was not accompanied by improvement in jump performance. It is likely the magnitude of inter-individual variation within the cohort of participants influenced the present findings to a large extent.

- Chapter 11 -

General Discussion

The series of studies comprising this thesis have endeavoured to add to the existing body of literature concerning the measurement of certain hormones in relation to exercise training, the response to high intensity exercise, and the potential influence of certain hormones on short-term performance.

Chapters 4, 5 and 6 investigated the agreement between the hormone concentrations measured through venous sampling and those in capillary and saliva samples, and the effect of different storage conditions and duration on the saliva hormone concentrations. In the first study to provide a comprehensive report on the agreement between venous and capillary concentrations over a wide array of hormones, good relation and agreement between venous and capillary concentrations of total testosterone, progesterone, estradiol, prolactin, FSH and LH was observed; suggesting that capillary sampling may be used as an acceptable alternative to venous sampling for these measures. However, correlation and agreement between venous concentrations of total and free IGF-1 and capillary equivalents was only moderate, suggesting that venous and capillary blood sampling may not be used interchangeably for determination of total and free IGF-1. For saliva sampling, although absolute LOA did not indicate direct agreement between venous and saliva measures, saliva hormone concentrations did display a proportional relationship with venous concentrations. Scaling of salivary concentrations revealed direct agreement between venous and saliva concentrations, suggesting that saliva samples can be used to track and reflect changes in venous hormone concentrations, but that appropriate correction

of saliva hormone concentrations is needed if saliva is to be used as an estimate of venous hormone concentrations of total cortisol and total testosterone.

In terms of the handling and storage of saliva samples, the data demonstrated that problems may arise when saliva is not frozen in the hours immediately after collection. While cortisol concentrations were not different between aliquots frozen immediately and those stored at room temperature or in a refrigerator for up to 7 days, a linear decrease in testosterone and estradiol concentrations was observed across 1 to 7 days of storage in non-freezer conditions. In order to preserve the 'original' concentrations of hormones in saliva, gonadal steroids should be kept refrigerated upon collection and stored at -20°C or below within 24 h. Samples to be analysed for cortisol may be stored or transported for up to 7 days at room temperature or in a refrigerator before freezing. Samples to be analysed for cortisol, testosterone or estradiol can remain frozen for up to 1 month prior to analysis. Establishing these guidelines for the handling and storage of samples following collection is an important finding.

Chapter 7 sought to assess the hormone responses to consecutive interval and resistance exercise bouts in men. In the male participants completing two sessions in the same day, performance of interval exercise in the morning resulted in increases in concentrations of cortisol, total testosterone and progesterone. Resistance exercise performed in the morning elicited an increase in the TC ratio while cortisol and prolactin concentrations decreased. When preceded by interval exercise, the magnitude of change in hormone concentration in response to resistance exercise in the afternoon was numerically greater for all hormones in comparison to completion

of resistance exercise in the morning, but only significant for cortisol and prolactin. The magnitude of change in concentration in response to interval exercise in the afternoon following completion of resistance exercise in the morning was varied, but was significantly greater for prolactin and the TC ratio . The data for IAUC and TAUC data suggested that it could be beneficial to perform resistance training in the afternoon preceded by interval training in the morning in order to stimulate a hormonal milieu that may be more conducive to increasing muscle protein turnover and increasing muscle mass.

Chapter 8 provided support for the hormone responses seen to the repeated sprint interval exercise bout performed in the morning. A cohort of both well-trained and recreationally active female participants completed the same repeated sprint interval exercise protocol and a robust increase was observed in circulating concentrations of testosterone, DHT and cortisol. DHT was analysed in this study following recent suggestions of its potential to exert both functional and signalling effects in skeletal muscle above those of testosterone (Hamdi & Mutungi, 2010). Similar to previous findings in males, cadence was associated with the magnitude of change in DHT concentration (Smith *et al.,* 2013), however, in contrast, the magnitude of change in testosterone was not. This may suggest that circulating DHT is synthesised via testosterone-independent pathways in females in response to exercise. It would be worthwhile for future investigations to establish whether DHT might be converted from local androgens within active muscle and role of DHT in acute athletic performance and chronic adaptation to exercise training.

Chapter 9 examined the influence of a sustained, high intensity, steady state cycling bout performed immediately prior, on the performance of, and hormone response to, the same repeated sprint interval exercise protocol used in the previous studies, as well as assessing proposed links between mood, hormone concentrations and performance. The prior exercise bout elicited an increase in total testosterone and DHT concentrations and a decrease in cortisol. Subsequent increases in concentrations of testosterone, DHT and cortisol in response to a repeated sprint cycle protocol performed immediately after were then observed suggesting no influence of the effort on the ability of the endocrine system to respond subsequent exercise, with magnitudes of change in testosterone and cortisol concentrations similar to those observed in Chapter 7. In terms of mood state, the data appeared to demonstrate some conflict. Small differences in affect were noted between trials following the 20 min effort, but were not associated with differences between trials in hormone concentrations or performance during the subsequent repeated sprint protocol, this may be suggestive of a disconnect in the association observed previously between mood, hormone concentrations and performance (Cook & Crewther 2012a; 2012b). However, across both trials, affect was positively correlated with total testosterone concentrations, and inversely associated with cortisol concentrations providing support for a possible link.

Chapter 10 endeavoured to further assess possible links between hormone concentrations and performance through investigating whether different methods of priming or potentiating exercise would be successful in enhancing power and strength performance, and if so, whether it was associated with elevations in hormone concentrations and muscular activation. No enhancement of vertical jump

performance or number of repetitions during performance of leg press exercise to fatigue with any of the potentiating exercises, suggesting postactivation potentiation did not occur. As such, no assessment of the possible association with hormone concentrations was made. However, muscular activation of the quadriceps during vertical jump performance was greater during the low cadence sprint and high cadence spints trials, and DHT and free testosterone increased in response to high cadence sprints. These data do not suggest a link between hormone concentrations and performance of explosive power or strength tasks, however, in support of the findings of Chapter 8, and previous research (Smith *et al.,* 2013), high cadence cycling generated an increase in concentrations of DHT.

The results of Chapters 4 & 5 provide evidence to support the use of both capillary and saliva sampling in an applied exercise setting for the majority of the hormones measured. While capillary hormone concentrations demonstrated absolute agreement with venous concentrations, the relationships established between venous and saliva concentrations suggest scaling of the data may be required. However, the necessity of this is dependent on the requirements of sampling. If hormone concentrations are to be used to track the pattern of change over a period of time using the same sample medium, scaling of concentrations may not be required. The results of Chapter 6 provide the basis from which to form recommendations for storage procedures in order to preserve sample integrity demonstrating samples should be refrigerated immediately and frozen within 24 h. In comparison to cortisol, testosterone appeared to be more unstable in saliva. In addition, the relationship observed in Chapter 5 between venous and saliva concentrations was weaker than observed for cortisol, reinforcing the need to maintain the integrity of the sample to avoid additional error.

Chapter 7 allowed for characterisation of the response to both interval and resistance exercise, and assessment of the influence of bout order on the responses. The data established demonstrate an effect of previous exercise on the cortisol response to resistance exercise following earlier interval exercise. These changes may have been related to ciracadian rhythm and feedback control, but raise an important consideration for the scheduling of training, especially in the light of the recent demonstrations of associations between concentrations of steroid hormones and athletic performance. In order to maximise training gains, athletes need to ensure they perform optimally during each session. Concentrations of steroid hormones may influence this.

The data presented in Chapter 7 also demonstrated a pronounced hormonal response to interval exercise, a finding that was mirrored in females in Chapter 8. Significant increases in testosterone, DHT and cortisol were accompanied by a positive association between the magnitude of change in DHT and cycling cadence. A observation that was also noted in Chapter 10. This may be suggestive of DHT being able to exert effect signalling and functional effects in skeletal muscle, as has been previously suggested (Hamdi & Mutungi, 2010). Cadence has also previously been demonstrated be related to the magnitude of change in testosterone concentration to the repeated sprint interval protocol in males (Smith *et al.,* 2013), however, this was not apparent in the female participants here. Synthesis of DHT can occur in a testosterone independent manner (Luu-The & Labrie, 2010), and given that females have an approximately 10 fold lower concentration of circulating testosterone than males (Vingren *et al.,* 2010), it is possible that production of DHT occurs more readily via testosterone independent pathways. As such, should DHT have the

signalling and functional effects proposed, in females, it may be a more important marker than testosterone in indicating adaptive potential and strength and power performance. However, this tentative suggestion requires further investigation.

Concerning the short term effects of hormones on performance, Chapters 9 & 10 sought to establish links between hormone concentrations and measures of mood and performance. The data in Chapter 9 demonstrated slightly conflicting evidence in regard to an association between hormone concentrations and mood. While slight differences observed in mood between trials were not related to differences in hormone concentration or performance, overall, a positive association was observed between affect and percentage change in testosterone. Despite prior performance of the pre-fatiguing cycling bout, increases in testosterone, DHT, and cortisol were observed in response to the repeated sprint cycling bout as used in Chapters 7 & 8. These data suggest no influence of the pre-fatigue effort on the ability of the endocrine system to respond subsequent exercise in this context. Magnitudes of increase in concentrations of cortisol and testosterone from pre- to post- exercise were similar to those observed in Chapter 7 following performance of the repeated sprint cycle bout alone.

In contrast to the potential associations identified between mood and hormone concentration in Chapter 9, no association between hormone concentrations and performance was observed in Chapter 10. The data observed did not demonstrate evidence of postactivation potentiation or an association between hormones concentrations and performance of strength and power tasks. The absence of this may have been related to the training status of the participants. Where participants are

recreationally trained, postactivation postentiation has been shown to be less effective (Chiu *et al.,* 2003; Khamoui *et al.,* 2009; Wilson *et al.,* 2013), and more highly trained individuals have been reported to demonstrate greater increases in androgens in response to exercise stimuli (Crewther *et al.,* 2006). Conversely, the data presented here may also indicate that the main mechanism by which androgens exert their effects on performance is through influencing mood and behaviour, as has recently been demonstrated (Cook and Crewther, 2012a; 2012b), and as aforementioned, demonstrated with an association in Chapter 9 between affect and change in testosterone concentration. However, the data did demonstrate a significant increase in DHT and free testosterone in response to high cadence cycling as was demonstrated in Chapter 8 and previously (Smith *et al.,* 2013). The precise mechanisms for this observation are unclear, but given recent evidence of increases in force production following DHT administration to muscle fibres in vitro (Hamdi & Mutungi, 2010), understanding this would seem to be an important step due to the possible implications for athletic performance.

In summary, the programme of research presented in this thesis has several interesting findings that may also form the basis for guidelines to inform practice within an applied exercise setting. These are stated below:

1. Capillary and saliva sampling can be used in the determination of concentrations of some hormones in an applied exercise setting. If a reflection of venous concentrations are required, saliva concentrations should be scaled accordingly.

2. Saliva samples should be refrigerated immediately upon collection, and frozen within 24 h. Subsequent analysis should take place within 28 days.

3. The order of training sessions can influence the hormone response elicited. In this context, it may be beneficial to perform resistance exercise in the afternoon in order to stimulate a hormonal milieu that may be more conducive to increasing muscle protein turnover.

4. Interval exercise elicits robust increases in testosterone, DHT and cortisol in females, with the magnitude of increase in DHT associated with sprinting cadence.

5. A prior period of high intensity cycling did not influence the ability of testosterone, DHT and cortisol to respond to a repeated sprint interval cycling bout, with the magnitude of change in testosterone positively associated with affect.

6. No associations were found between hormone concentrations and strength and power performance following no observation of a postactivation potentiation effect to different exercise stimuli. However increases in DHT and free testosterone were observed in response to high cadence cycling.

While the research presented here contributes to the existing body of knowledge regarding the measurement of circulating hormone concentrations, their response to high intensity exercise, and the potential influence on short-term performance, it also demonstrates potential future avenues for research. Two observations were made over the course of this research of an association between DHT concentrations and high

cadence cycling. An observation that has also previously been reported (Smith *et al.*, 2013). Given the reported influence of DHT on muscle force production in vitro, there may be possible implications for performance. In addition, it would be of interest to further elucidate the influence of bout order of different modes of exercise on hormone responses and performance.

- References -

References

Acevedo, E. O., Kraemer, R. R., Kamimori, G. H., Durand, R. J., Johnson, L. G. & Castracane, D. V. (2007). Stress hormones, effort sense, and perceptions of stress during incremental exercise: an exploratory investigation. *Journal of Strength and Conditioning Research*, 21(1): 283-288.

Ahn, R. S., Lee, Y. J., Choi, J. Y., Kwon, H. B. & Chun, S. I. (2007). Salivary cortisol and dhea levels in the korean population: Age-related differences, diurnal rhythm, and correlations with serum levels. *Yonsei Medical Journal,* 48: 379-88.

Ahtiainen, J. P., Pakarinen, A., Alen, M., Kraemer, W. J. & Hakkinen, K. (2003). Muscle hypertrophy, hormonal adaptations and strength development during strength training in strength-trained and untrained men. *European Journal of Applied Physiology,* 89: 555-563.

Ahtiainen, J. P., Pakarinen, A., Alen, M., Kraemer, W. J. & Hakkinen, K. (2005). Short vs. Long rest period between the sets in hypertrophic resistance training: Influence on muscle strength, size and hormonal adaptations in trained men. *Journal of Strength and Conditioning Research,* 19: 572-582.

Aizawa, K., Iemitsu, M., Maeda, S., Otsuki, T., Sato, K., Ushida, T., Mesaki, N. & Akimoto, T. (2010). Acute exercise activates local bioactive androgen metabolism in skeletal muscle. *Steroids,* 75: 219–223.

Aleman, A., Bronk, E., Kessels, R.P., Koppeschaar, H.P. & van Honk J. (2004). A single administration of testosterone improves visuospatial ability in young women. *Psychoneuroendocrinology,* 29: 612–617.

Archer J. (2006). Testosterone and human aggression: an evaluation of the challenge hypothesis. *Neuroscience & Biobehavioral Review,* 30: 319–345.

Arregger, A.L., Contreras, L.N., Tumilasci, O.R., Aquilano, D.R. & Cardoso, E.M. (2007). Salivary testosterone: A reliable approach to the diagnosis of male hypogonadism. *Clin Endocrinology (Oxf),* 67: 656-62.

Atkinson, G., & Nevill, A. M. (1998). Statistical methods for assessing measurement error (reliability) in variables relevant to sports medicine. *Sports Medicine,* 26(4): 217-238.

Bauer, E.R., Daxenberger, A., Petri, T., Sauerwein, H. & Meyer, H.H. (2000). Characterisation of the affinity of different anabolics and synthetic hormones to the human androgen receptor, human sex hormone binding globulin and to the bovine progestin receptor. *APMIS,* 108: 838–846,

Beaven, C.M., Cook, C.J. & Gill, N.D. (2008a). Significant strength gains observed in rugby players after specific resistance exercise protocols based on individual salivary testosterone responses. *Journal of Strength and Conditioning Research,* 22: 419-25.

Beaven, C.M., Gill, N.D. & Cook, C.J. (2008b). Salivary testosterone and cortisol responses in professional rugby players after four resistance exercise protocols. *Journal of Strength and Conditioning Research,* 22: 426-32.

Bell, G. J., Syrotuik, D., Martin, T. P., Burnham, R. & Quinney, H. A. (2000). Effect of concurrent strength and endurance training on skeletal muscle properties and hormone concentrations in humans. *European Journal of Applied Physiology,* 81: 418-27.

Berchtold M. W., Brinkmeier, H. & Muntener, M. (2000). Calcium ion in skeletal muscle: its crucial role for muscle function, plasticity, and disease. *Physiology Reviews*, 80 (3): 1215-65

Bird, S. P. & Tarpenning, K. M. (2004). Influence of circadian time structure on acute hormonal responses to a single bout of heavy-resistance exercise in weight-trained men. *Chronobiology International,* 21: 131-46.

Bishop, N. C., Gleeson, M. & Nicholas, C. W. (2002). Influence of carbohydrate supplementation on plasma cytokine and neutrophil degranulation responses to high intensity intermittent exercise, I*nternational Journal of Sports Nutrition and Exercise Metabolism,* 12: 145 – 156.

Bland, J. M., & Altman, D. G. (2007). Agreement between methods of measurement with multiple observations per individual. *Journal of Biopharmacuetical Statistics,* 17(4): 571-582.

Bloomer, R. J. & Farney, T. M. (2013). Acute plasma volume change with high intensity sprint exercise. *Journal of Strength and Conditioning Research*, 27(10): 2874 - 2878.

Bonifazi M., Ginanneschi, F., della Volpe R. & Rossi A. (2004). Effects of gonadal steroids on the input-output relationship of the corticospinal pathway in humans. *Brain Research,* 1011: 187–194.

Bosco, C., Colli, R., Bonomi, R., von Duvillard, S. P. & Viru, A. (2000). Monitoring strength training: Neuromuscular and hormonal profile. *Medicine and Science in Sports and Exercise,* 32: 202-8.

Brandenberger, G., Follenius, M. & Muzet, A. (1984). Interactions between spontaneous and provoked cortisol secretory episodes in man. *Journal of Clinical Endocrinology and Metabolism,* 59: 406-11.

Braun, B. & Horton, T. (2001). Endocrine regulation of exercise substrate utilisation in women compared to men. *Exercise and Sport Science Reviews*, 29(4): 149-154.

Cadore, E. L., Izquierdo, M., dos Santos, M. G., Martins, J. B., Rodrigues Lhuiller, F. L., Pinto, R. S., Silva, R. F. & Kruel, L. F. M. (2012). Hormonal responses to concurrent strength and endurance training with different exercise orders. Journal of Strength and Conditioning Reasearch, 26(12): 3281-3288.

Cardinale, M. & Stone, M. H. (2006). Is testosterone influencing explosive performance? *Journal of Strength and Conditioning Research*, 20(1): 103-107.

Chikuma, T., Hanaoka, K., Loh, Y.P., Kato, T. & Ishii, Y. (1991). A colorimetric assay for measuring peptidylglycine alpha-amidating monooxygenase using high-performance liquid chromatography. *Anal Biochem,* 198: 263-7.

Chiu, L. Z. F., Fry, A. C., Weiss, L. W., Schilling, B. K., Brown, L. E. & Smith, S. (2003). Postactivation Potentiation Response in Athletic and Recreationally Trained Individuals. *Journal of Strength and Conditioning Research,* 17(4): 671–677.

Chtara, M., Chaouachi, A., Levin, G. T., Chaouachi, M., Chamari, K., Amri, M. & Laursen, P. B. (2008). Effect of concurrent endurance and circuit resistance training sequence on muscular strength and power development. *Journal of Strength and Conditioning Research,,* 22: 1037-45.

Consitt, L.A., Copeland, J.L. & Tremblay, M.S. (2002). Endogenous anabolic hormone responses to endurance versus resistance exercise and training in women. *Sports Medicine,* 32: 1-22.

Cook, C. J. & Crewther, B. T. (2012a). Changes in salivary testosterone concentrations and subsequent voluntary squat performance following the presentation of short video clips. *Hormones & Behaviour,* 61: 17–22.

Cook, C.J , Crewther, B. T. (2012b). The effects of different pre-game motivational interventions on athlete free hormonal state and subsequent performance in professional rugby union matches. *Physiology & Behaviour,*106: 683–688.

Cormack, S. J., Newton, R. U., McGuigan, M. R. & Doyle, T. L. A. (2008). Reliability of Measures Obtained During Single and Repeated Countermovement Jumps. *International Journal of Sports Physiology and Performance,* 3: 131-144

Crawford, D. E., Barqawi, A.B., O'Donnell, C. & Morgentaler, A. (2007). The association of time of day and serum testosterone concentration in a large screening population. *Urological Oncology,* 100: 509-513.

Crewther, B., Cronin, J., Keogh, J. & Cook, C. (2008). The salivary testosterone and cortisol response to three loading schemes. *Journal of Strength and Conditioning Research,* 22: 250-5.

Crewther, B., Keogh, J., Cronin, J., & Cook, C. (2006). Possible stimuli for strength and power adaptation: acute hormonal responses. *Sports Medicine,* 36(3): 215-238.

Crewther, B. T., & Cook, C. (2010). Measuring the salivary testosterone and cortisol concentrations of weightlifters using an enzyme-immunoassay kit. *International Journal of Sports Medicine,* 31(7): 486-489.

Crewther, B. T., Cook, C. J., Lowe, T. E., Weatherby, R. P. & Gill, N. (2011a). The effects of short-cycle sprints on power, strength, and salivary hormones in elite rugby players. *Journal of Strength and Conditioning Research*, 25(1): 32–39.

Crewther, B. T., Kilduff, L. P., Cook, C. J., Middleton, M. K., Bunce, P. J. & Yang, G. Z. (2011b). The acute potentiating effects of back squats on athlete performance. *Journal of Strength and Conditioning Research*, 25(12): 3319–3325.

Crewther, B. T., Cook, C. J., Cardinale, M., Weatherby, R. P. & Lowe, T. (2011c). Two emerging concepts for elite athletes: the short-term effects of testosterone and cortisol on the neuromuscular system and the dose-response training role of these endogenous hormones. *Sports Medicine,* 41: 103–123.

Crewther, B. T., Kilduff, L. P., Cunningham, D. J., Cook, C., Owen, N. & Yang, G. Z. (2011d). Validating two systems for estimating force and power. *International Journal of Sports Medicine*, 32: 254 - 258.

Crewther, B. T., Cook, C. J., Gaviglio, C. M., Kilduff, L. P. & Drawer, S. (2012a). Baseline strength can influence the ability of salivary free testosterone to predict squat

and sprinting performance. *Journal of Strength & Conditioning Research,* 26: 261–268.

Crewther, B. T., Kilduff, L. P., Cook, C. J., Cunningham, D. J., Bunce, P., Bracken, R. M. & Gaviglio, C. M. (2012b). Relationships between salivary free testosterone and the expression of force and power in elite athletes. *Journal of Sports Medicine &Physical Fitness,* 52: 221–227.

Crewther, B. T., Lowe, T. E., Ingram, J., & Weatherby, R. P. (2010). Validating the salivary testosterone and cortisol concentration measures in response to short high-intensity exercise. *Journal of Sports Medicine and Physical Fitness,* 50(1): 85-92.

Crewther, B. T., Lowe, T., Weatherby, R. P., Gill, N. & Keogh, J. (2009). Neuromuscular performance of elite rugby union players and relationships with salivary hormones. *Journal of Strength and Conditioning Research*, 23(7): 2046–2053.

Cronin, J. B., Hing, R. D. & McNair, P. J. (2004). Reliability and validity of a linear position transducer for measuring jump performance. *Journal of Strength and Conditioning Research*, 18(3): 590-593.

Dalbo, V. J., Roberts, M. D., Hassell, S. E., Brown, R. D. & Kerksick, C. M. (2011). Effects of age on serum hormone concentrations and intramuscular proteolytic signalling before and after a single bout of resistance training, *Journal of Strength and Conditioning Research,*, 25: 1-9.

Daly, W., Seegers, C. A., Rubin, D. A., Dobridge, J. D. & Hackney, A. C. (2005). Relationship between stress hormones and testosterone with prolonged endurance exercise. *European Journal of Applied Physiology,* 93: 375-80.

De la Torre, R., de la Torre, X., Alia, C., Segura, J., Baro, T. & Torres-Rodriguez, J.M. (2001). Changes in androgenic steroid profile due to urine contamination by microorganisms: A prospective study in the context of doping control. *Analytical Biochemistry,* 289: 116-23.

Diver, M. J., Imtiaz, K. E., Ahmad, A. M., Vora, J. P. & Fraser, W. D. (2003). Diurnal rhythms of serum total, free and bioavailable testosterone and of shbg in middle-aged men compared with those in young men. *Clinical Endocrinology (Oxf),* 58: 710-7.

Docherty, D. & Sporer, B. (2000). A proposed model for examining the interference phenomenon between concurrent aerobic and strength training. *Sports Medicine,* 30: 385-94.

Duclos, M., Corcuff, J. B., Arsac, L., Moreau-Gaudry, F., Rashedi, M., Roger, P., Tabarin, A. & Manier, G. (1998). Corticotroph axis sensitivity after exercise in endurance-trained athletes. *Clinical Endocrinology (Oxf),* 48: 493-501

Edwards, D.A., Wetzel, K. & Wyner, D.R. (2006). Intercollegiate soccer: Saliva cortisol and testosterone are elevated during competition, and testosterone is related to status and social connectedness with team mates. *Physiology and Behaviour,* 87: 135-43.

Eliakim, A. & Nemet, D. (2010). Exercise training, physical fitness, and the growth hormone – insulin-like growth factor-1 axis and cytokine balance. *Medicine and Sport Science,* 55: 128 – 140.

Elloumi, M., Maso, F., Michaux, O., Robert, A. & Lac, G. (2003). Behaviour of saliva cortisol [c], testosterone [t] and the t/c ratio during a rugby match and during the post-competition recovery days. *European Journal of Applied Physiology,* 90: 23-8.

Estrada, M., Espinosa, A., Muller, M., et al. (2003). Testosterone stimulates intracellular calcium release and mitogenactivated protein kinases via a Gprotein coupled receptor in skeletal muscle cells. *Endocrinology,* 144 (8): 3586-97

Fair, A.M. & Montgomery, K. 2009. Energy balance, physical activity, and cancer risk. *Methods in Molecular Biology,* 472: 57 – 88.

Ferrando, A. A., Sheffield-Moore, M., Yeckel, C. W., Gilkison, C., Jiang, J., Achacosa, A., Lieberman, S. A., Tipton, K., Wolfe, R. R. & Urban, R. J. (2002). Testosterone administration to older men improves muscle function: molecular and physiological mechanisms. *American Journal of Physiology Endocrinology and Metabolism,* 282: E601–E607.

Filaire, E., Bernain, X., Sagnol, M. & Lac, G. (2001). Preliminary results on mood state, salivary testosterone:Cortisol ratio and team performance in a professional soccer team. *European Journal of Applied Physiology,* 86: 179-84.

Firth, S. M. & Baxter, R. C. (2002) Cellular actions of the insulin-like growth factor binding proteins. *Endocrine Review,* 23: 824 – 854.

Fitzgerald, R. L., Griffin, T. L. & Herold, D. A. (2010). Analysis of testosterone in serum using mass spectrometry. *Methods in Molecular Biology*, 603: 489-500.

Fryer, S., Hillier, S., Dickson, T., Draper, N., Stoner, L., Winter, D., Young, J. & Cohen, L. (2012). Capillary cortisol sampling during high intensity exercise. *International Journal of Sports Medicine,* 33(10): 842-845.

Frystyk, J. (2004). Free insulin like growth factors – measurements and relationships to growth hormone secretion and glucose homeostasis, PhD thesis

Frystyk, J., Freda, P. & Clemmons, D. R. (2010). The current status of igf-i assays--a 2009 update. *Growth Hormone & IGF Research.* 20: 8-18.

Garde, A.H. & Hansen, A.M. (2005). Long-term stability of salivary cortisol. *Scandinavian Journal of Clinical Laboratory Investigation,* 65: 433-6.

Gatti, R. & De Palo, E.F. (2011). An update: Salivary hormones and physical exercise. *Scandinavian Journal of Medicine and Science in Sports*, 21: 157-169.

Girard, O. & Racinais, S. (2014). Combining heat stress and moderate hypoxia reduces cycling time to exhaustion without modifying neuromuscular fatigue characteristics. *European Journal of Applied Physiology*, 114: 1521-1532.

Godfrey, R. J., Whyte, G., McCarthy, J., Nevill, A., & Head, A. (2004). The validity of capillary blood sampling in the determination of human growth hormone concentration during exercise in men. *British Journal of Sports Medicine,* 38(5): E27.

Godoy, A., Kawinski, E., Li, Y., Oka, D., Alexiev, B., Azzouni, F., Titus, M.A. & Mohler, J.L. (2011). 5α-Reductase type 3 expression in human benign and malignant tissues: a comparative analysis during prostate cancer progression. *Prostate* 71: 1033–1046.

Gonzalez-Alonso, J., Teller, C., Andersen, S., Jensen, F., Hyldig, T. & Nielsen B. (1999). Influence of body temperature on the development of fatigue during prolonged exercise in the heat. *Journal of Applied Physiology*, 86: 1032-1039.

Goto, K., Higashiyama, M., Ishii, N. & Takamatsu, K. (2005). Prior endurance exercise attenuates growth hormone response to subsequent resistance exercise. *European Journal of Applied Physiology,* 94: 333-8.

Goto, K., Ishii, N., Kurokawa, K. & Takamatsu, K. (2007). Attenuated growth hormone response to resistance exercise with prior sprint exercise. *Medicine and Science in Sports and Exercise,* 39: 108-15.

Gozansky, W. S., Lynn, J. S., Laudenslager, M. L., & Kohrt, W. M. (2005). Salivary cortisol determined by enzyme immunoassay is preferable to serum total cortisol for assessment of dynamic hypothalamic--pituitary--adrenal axis activity. *Clinical Endocrinology (Oxf),* 63(3): 336-341.

Granger, D. A., Kivlighan, K. T., Fortunato, C., Harmon, A. G., Hibel, L. C., Schwartz, E. B., et al. (2007). Integration of salivary biomarkers into developmental and behaviorally-oriented research: problems and solutions for collecting specimens. *Physiology and Behaviour,* 92(4): 583-590.

Granger, D. A., Shirtcliff, E. A., Booth, A., Kivlighan, K. T. & Schwartz, E. B. (2004). The "trouble" with salivary testosterone. *Psychoneuroendocrinology,* 29, 1229-40.

Grino, P.B., Griffin, J.E. & Wilson, J.D. (1990). Testosterone at high concentrations interacts with the human androgen receptor similarly to dihydrotestosterone. *Endocrinology* 126: 1165–1172.

Groschl, M. (2008). Current status of salivary hormone analysis. *Clinical Chemistry,* 54: 1759-69.

Groschl, M., Wagner, R., Rauh, M. & Dorr, H. G. (2001). Stability of salivary steroids: The influences of storage, food and dental care. *Steroids,* 66: 737-41.

Hakkinen, K., Alen, M., Kraemer, W. J., Gorostiaga, E., Izquierdo, M., Rusko, H., Mikkola, J., Hakkinen, A., Valkeinen, H., Kaarakainen, E., Romu, S., Erola, V., Ahtiainen, J. & Paavolainen, L. (2003). Neuromuscular adaptations during concurrent strength and endurance training versus strength training. *European Journal of Applied Physiology,* 89: 42-52.

Hakkinen, K. & Pakarinen, A. (1993). Acute hormonal responses to two different fatiguing exercise protocols in male athletes. *Journal of Applied Physiology,* 74: 882-887.

Hamada, T., Sale, D. G., MacDougall, J. D., et al. (2003). Interaction of fibre type, potentiation and fatigue in human knee extensor muscles. *Acta Physiologica Scandinavica*, 178 (2): 165-73.

Hamdi, M. M. & Mutungi, G. (2010). Dihydrotestosterone activates the MAPK pathway and modulates maximum isometric force through the EGF receptor in isolated intact mouse skeletal muscle fibres. *Journal of Physiology,* 588: 511–525.

Han, J. Z., Lin, W., Chen, Y. Z. (2005). Inhibition of ATP-induced calcium influx in HT4 cells by glucocorticoids: involvement of protein kinase A. Acta Pharmalogica Scandinavica, Sinic, 26 (2): 199-204.

Hautier, C. A., Linossier, M. T., Belli, A., Lacour, J. R. & Arsac, L. M. (1996). Optimal velocity for maximal power production in non-isokinetic cycling is related to muscle fibre type composition. *European Journal of Applied Physiology & Occupational Physiology,* 74: 114–118.

Hawkins, V. N., Foster-Schubert, K., Chubak, J., Sorensen, B., Ulrich, C. M., Stancyzk, F. Z., Plymate, S., Stanford, J., White, E., Potter, J. D. & McTiernan, A. (2008). Effect of exercise on serum sex hormones in men: a 12-month randomized clinical trial. *Medicine & Science in Sports& Exercise* 40: 223–233.

Helge, J. W., Stallknecht, B., Pedersen, B. K., Galbo, H., Kiens, B., & Richter, E. A. (2003). The effect of graded exercise on IL-6 release and glucose uptake in human skeletal muscle, *Journal of Physiology,* 546: 299 – 305.

Hodgson, M., Docherty, D. & Robbins, D.(2005). Post-Activation Potentiation: Underlying Physiology and Implications for Motor Performance. *Sports Medicine,* 35 (7): 585-595.

Hodgson, M.J., Docherty, D. & Zehr, P.E. (2008). Postactivation Potentiation of Force Is Independent of H-Reflex Excitability. *International Journal of Sports Physiology and Performance,* 3: 219-231.

Izquierdo, M., Hakkinen, K., Ibanez, J., Garrues, M., Anton, A., Zuniga, A., Larrion, J. L. & Gorostiaga, E. M. (2001). Effects of strength training on muscle power and serum hormones in middle-aged and older men. *Journal of Applied Physiology,* 90: 1497-507.

Izquierdo, M., Ibanez, J., Calbet, J.A., Navarro-Amezqueta, I., Gonzalez-Izal, M., Idoate, F., Hakkinen, K., Kraemer, W.J., Palacios-Sarrasqueta, M., Almar, M. &

Gorostiaga, E. M. (2009). Cytokine and hormone responses to resistance training. *European Journal of Applied Physiology*, 107: 397-409.

Izquierdo, M., Ibanez, J., Hakkinen, K., Kraemer, W.J., Ruesta, M. & Gorostiaga, E.M. (2004). Maximal strength and power, muscle mass, endurance and serum hormones in weightlifters and road cyclists. *Journal of Sports Sciences,* 22: 465-478.

Jaimovich, E. & Espinosa, A. (2004). Possible link of different slow calcium signals generated by membrane potential and hormones to differential gene expression in cultured muscle cells. *Biological Research,* 37 (4): 625-33.

Jurimae, J., Nurmekivi, A. & Jurimae, T. (2004). Hormone responses to intensive interval training in middle-distance runners. *Biology of Sport,* 21: 67-79.

Juul, A. (2003). Serum levels of insulin-like growth factor i and its binding proteins in health and disease. *Growth Hormone & IGF Research,* 13: 113-70.

Kanaley, J. A., Weltman, J. Y., Pieper, K. S., Weltman, A. & Hartman, M. L. (2001). Cortisol and growth hormone responses to exercise at different times of day. *Journal of Clinical Endocrinology & Metabolism,* 86: 2881-9.

Kato, T., Hanaoka, K., Ochiai, O., Chikuma, T., Takeda, K., Yajima, R. & Kumegawa, M. (1992). Secretion of peptidylglycine alpha-amidating monooxygenase (pam) from rat salivary glands. *Neurochemistry International,* 21: 153-8.

Kaufmann, G., Dautzenberg, H., Henkel, H., Muller, G., Schafer, T., Undeutsch, B. & Oettel, M. (1999). Nitrile hydratase from rhodococcus erythropolis: Metabolization of steroidal compounds with a nitrile group. *Steroids,* 64: 535-40.

Keizer, H. A., Beckers, E., de Haan, J., Janssen, G. M., Kuipers, H., van Kranenburg, G. & Geuten, P. (1987). Exercise-induced changes in the percentage of free testosterone and estradiol in trained an untrained women. *International Journal of Sports Medicine*, 8(3): 151-153.

Khamoui, A.V., Brown, L. E., Coburn, J. W., Judelson, D. A., Uribe, B. P., Nguyen, D., Tran, T., Eurich, A. D. & Noffal, G. J. (2009). Effect of potentiating exercise volume on vertical jump parameters in recreationally trained men. *Journal of Strength and Conditioning Research*, 23(5): 1465–1469.

Kilduff, L. P., Bevan, H. R., Kingsley, M. I. C, Owen, N. J., Bennett, M. A., Bunce, P. J., Hore, A. M., Maw, J. R. & Cunningham, D. J. (2007). Postactivation potentiation in professional rugby players: optimal recovery. *Journal of Strength and Conditioning Research*, 21(4): 1134 - 1138.

Kilduff, L. P., Owen, N., Bevan, H., Bennett, M., Kingsley, M. I. C. & Cunningham, D. (2008). Influence of recovery time on post-activation potentiation in professional rugby players. *Journal of Sports Sciences,* 26(8): 795 - 802.

Kilduff, L.P., West, D. J., Williams, H. & Cook C. J. (2013). The influence of passive heat maintenance on lower body power output and repeated sprint performance in professional rugby league players. *Journal of Science and Medicine in Sport*, 16: 482 - 486.

Kivlighan, K.T., Granger, D.A., Schwartz, E.B., Nelson, V., Curran, M. & Shirtcliff, E.A. (2004). Quantifying blood leakage into the oral mucosa and its effects on the measurement of cortisol, dehydroepiandrosterone, and testosterone in saliva. *Hormones & Behaviour,* 46: 39-46.

Kostek, M. C., Delmonico, M. J., Reichel, J. B., Roth, S. M., Douglass, L., Ferrell, R. E. & Hurley, B. F. (2005). Muscle strength response to strength training is influenced by insulin-like growth factor 1 genotype in older adults. *Journal of Applied Physiology,* 98: 2147-54.

Kraemer, R. R., Hollander, D. B., Reeves, G. V., Francois, M., Ramadan, Z. G., Meeker, B., Tryniecki, J. L., Hebert, E. P. & Castracane, V. D. (2006). Similar hormonal responses to concentric and eccentric muscle actions using relative loading. *European Journal of Applied Physiology*, 96: 551-7.

Kraemer, W. J., French, D. N., Paxton, N. J., Häkkinen, K., Volek, J. S., Sebastianelli, W. J., Putukian, M., Newton, R. U., Rubin, M. R., Gómez, A. L., Vescovi, J. D., Ratamess, N. A., Fleck, S. J., Lynch, J. M. & Knuttgen, H. G. (2004). Changes in exercise performance and hormonal concentrations over a big ten soccer season in starters and nonstarters. *Journal of Strength and Conditioning Research*, 18(1): 121-8.

Kraemer, W. J., Hakkinen, K., Newton, R. U., Nindl, B. C., Volek, J. S., McCormick, M., Gotshalk, L. A., Gordon, S. E., Fleck, S. J., Campbell, W. W., Putukian, M. & Evans, W. J. (1999). Effects of heavy resistance training on hormonal response patterns in younger vs. older men. *Journal of Applied Physiology,* 87 (3): 982-92

Kraemer, W. J., Marchitelli, L. J., Gordon, S. E., Harman, E. A., Dziados, J. E., Mello, R., et al. (1990). Hormonal and growth factor responses to heavy resistance exercise protocols. *Journal of Applied Physiology,* 69(4): 1442-1450.

Kraemer, W. J., & Ratamess, N. A. (2005). Hormonal responses and adaptations to resistance exercise and training. *Sports Medicine,* 35(4): 339-361.

Kvorning, T., Andersen, M., Brixen, K. & Madsen, K. (2006). Suppression of endogenous testosterone production attenuates the response to strength training: a randomised placebo-controlled and blinded intervention study. *American Journal of Physiology Endocrinology & Metabolism*, 291(6): E1325 - E1332.

Lac, G. (2001). Saliva assays in clinical and research biology. *Pathologie-Biologie (Paris),* 49(8): 660-667.

Lac, G., Lac, N., & Robert, A. (1993). Steroid assays in saliva: a method to detect plasmatic contaminations. *Archives Internationale de Physiologie, de Biochimie et de Biophysique,* 101(5): 257-262.

Lapauw, B., Goemaere, S., Crabbe, P., Kaufman, J. M. & Ruige, J. B. (2007). Is the effect of testosterone on body composition modulated by the androgen receptor gene cag repeat polymorphism in elderly men? *European Journal of Endocrinology,* 156: 395-401.

Laudat, M. H., Cerdas, S., Fournier, C., Guiban, D., Guilhaume, B., & Luton, J. P. (1988). Salivary cortisol measurement: a practical approach to assess pituitary-adrenal function. *Journal of Clinical Endocrinology and Metabolism,* 66(2): 343-348.

Lopez-Alarcon, M., Hunter, G. R., Gower, B. A. & Fernandez, J. R. (2007). Igf-i polymorphism is associated with lean mass, exercise economy, and exercise performance among premenopausal women. *Archives of Medical Research,* 38: 56-63.

Lu, S. S., Lau, C. P., Tung, Y. F., Huang, S. W., Chen, Y. H., Shih, H. C., Tsai, S. C., Lu, C. C., Wang, S. W., Chen, J. J., Chien, E. J., Chien, C. H. & Wang, P. S. (1997). Lactate and the effects of exercise on testosterone secretion: Evidence for the involvement of a camp-mediated mechanism. *Medicine and Science in Sports and Exercise,* 29: 1048-54.

Luu-The, V. & Labrie, F. (2010). The intracrine sex steroid biosynthesis pathways. *Progress in Brain Research*, 181:177-192.

Macarthur, D. G. & North, K. N. (2005). Genes and human elite athletic performance. *Human Genetics,* 116: 331-9.

Macpherson, L. M. & Dawes, C. (1999). Urea concentration in minor mucous gland secretions and the effect of salivary film velocity on urea metabolism by streptococcus vestibularis in an artificial plaque. *Journal of Periodontal Research,* 26: 395-401.

Maloney, S. J., Turner, A. N., & Fletcher, I. M. (2014). Ballistic exercises as a pre-activation stimulus: a review of the literature and practical applications. *Sports Medicine*, 44: 1347 - 1359.

Mareck, U., Geyer, H., Opfermann, G., Thevis, M. & Schanzer, W. (2008). Factors influencing the steroid profile in doping control analysis. *Journal of Mass Spectrometry,* 43: 877-91.

Maresh, C. M., Sokmen, B., Armstrong, L. E., Dias, J.C, Pryor, J. L., Creighton, B. C., Muñoz, C. X., Apicella, J. M., Casa, D. J., Lee, E. C., Anderson, J. M. & Kraemer, W. J. (2014). Repetitive box lifting performance in impaired in a hot environment: implications for altered work-rest cycles. *Journal of Occupational and Environmental Hygiene*, 11(7): 460-468.

McCall, G. E., Byrnes, W.C., Fleck, S. J., Dickinson, A. & Kraemer, W. J. (1999). Acuteand chronic hormonal responses to resistance training designed to promote muscle hypertrophy. *Canadian Journal of Applied Physiology,* 24: 96–107.

McMillan J. L., Stone M. H., Sartin J., et al. (1993). 20-hour physiological responses to a single weight-training session. *Journal of Strength and Conditioning Research,* 7 (1): 9-21

Meckel, Y., Eliakim, A., Seraev, M., Zaldivar, F., Cooper, D. M., Sagiv, M. & Nemet, D. (2009). The effect of a brief sprint interval exercise on growth factors and inflammatory mediators. *Journal of Strength and Conditioning Research,* 23: 225-30.

Meckel, Y., Nemet., D. & Bar-Sela, S. (2011). Hormonal and inflammatory responses to different types of sprint interval training, *Journal of Strength and Conditioning Research*, 25: 2161 – 2169.

Meeusen, R., Nederhof, E., Buyse, L., Roelands, B., de Schutter, G. & Piacentini, M. F. (2010). Diagnosing overtraining in athletes using the two-bout exercise protocol. *British Journal of Sports Medicine*, 44(9): 642-648.

Mertens, H. J. M. M., Heineman, M. J., Theunissen, P. H. M. H., de Jong, F. H. & Evers, J. L. H. (2001). Androgen, estrogen and progesterone receptor expression in the human uterus during the menstrual cycle. *European Journal of Obstetrics and Gymaecology and Reproductive Biology*, 98: 58 - 65.

Newton, R. U., Kraemer, W. J., Hakinnen, K., Humphries, B. J. & Murphy, A. J. (1996). Kinematics, kinetics, and muscle activation during explosive upper body movements. Journal of Applied Biomechanics, 12(1): 31 - 43.

Nibali, M. L., Chapman, D. W., Robergs, R. A. & Drinkwater, E. J. (2013). Validation of jump squats as a practical measure of post-activation potentiation. *Applied Physiology Nutrition & Metabolism*, **38**: 306–313.

Nielsen, B., Hyldig, T., Bidstrup, F. & Gonzalez-Alonso, J. (2001). Brain activity and fatigue during prolonged exercise in the heat. *Pflugers Archive*, 442: 41-48.

Nindl, B. C., Alemany, J. A., Kellogg, M. D., Rood, J., Allison, S. A., Young, A .J. & Montain, S. J. (2007). Utility of circulating IGF-1 as a biomarker for assessing body composition changes in men during periods of high physical activity superimposed upon energy and sleep restriction. *Journal of Applied Physiology*, 103: 340-346.

Nindl B. C., Kellogg, M. D., Khosravi, M. J., Diamandi, A., Alemany, J. A., Pietila, D. M., Young, A. J., & Montain, S. J. (2003). Measurement of insulin-like growth factor I during military operational stress via a filter paper blood spot assay. *Diabetes Technology & Therapeutics,* 5(3): 455 – 461.

Nguyen T. V., Yao, M. & Pike, C. J. (2005) Androgens activate mitogen activated protein kinase signalling: role in neuroprotection. *Journal of Neurochemistry,* 94 (6): 1639-51

Opaszowski, B. H. & Busko, K. (2003). Plasma growth hormone, cortisol and testosterone responses to repeated and intermittent ergometer exercise at different pedalling rates. *Biology of Sport,* 20: 159-172.

Paccotti, P., Minetto, M., Terzolo, M., Ventura, M., Ganzit, G. P., Borrione, P., et al. (2005). Effects of high-intensity isokinetic exercise on salivary cortisol in athletes with different training schedules: relationships to serum cortisol and lactate. *International Journal of Sports Medicine,* 26(9): 747-755.

Papacosta, E., & Nassis, G. P. (2011). Saliva as a tool for monitoring steroid, peptide and immune markers in sport and exercise science. *Journal of Science and Medicine in Sport,* 14(5): 424-434.

Pardridge, W. M. (1986). Serum bioavailability of sex steroid hormones. *Clinical Endocrinology and Metabolism,* 15(2): 259-278.

Passaquin, A. C., Lhote, P. & Ruegg U. T. (1998) Calcium influx inhibition by steroids and analogs in C2C12 skeletal muscle cells. *British Journal of Pharmacology,* 124 (8): 1751-9.

Peake, J., Wilson, G., Mackinnon, L., & Coombes, J. S. (2005). Carbohydrate supplementation and alterations in neutrophils, and plasma cortisol and myoglobin concentration after intense exercise. *European Journal of Applied Physiology,* 93(5-6): 672-678.

Pullinen, T., MacDonald, E., Pakarinen, A., Komi, P. V. & Mero, A. (2005). Hormonal responses and muscle fatigue in maximal repetitive sprinting. *Journal of Human Movement Studies,* 48, 91-107.

Reed, G. F., Lynn, F. & Meade, B. D. (2002). Use of coefficient of variation in assessing variability of quantitative assays. *Clinical and Diagnostic Laboratory Immunology,* 9: 1235-9.

Roberts, M. D., Dalbo, V. J., Hassell, S. E. & Kerksick, C. M. (2009). The expression of androgen-regulated genes before and after a resistance exercise bout in younger and older men. *Journal of Strength and Conditioning Research*, 23: 1060-7.

Rojas Vega, S., Knicker, A., Hollmann, W., Block, W. & Struder, H. K. (2010). Effect of resistance exercise on serum levels of growth factors in humans, *Hormone and Metabolism Research.,* 42: 982 – 986.

Ronsen, O., Haug, E., Pedersen, B. K. & Bahr, R. (2001). Increased neuroendocrine response to a repeated bout of endurance exercise. *Medicine and Science in Sports and Exercise,* 33: 568-75.

Rosa, C., Vilaca-Alves, J., Fernandes, H. M., Saavedra, F. J., Pinto, R. S. & Dos Reis, V. M. (2014). Order effects of combined strength and endurance training on testosterone, cortisol. growth hormone and IGF-1 binding protein 3 in concurrently trained men. *Journal of Strength and Conditioning Research*, e-pub before print.

Rothman, M. S., Carlson, N. E., Xu, M., Wang, C., Swerdloff, R., Lee, P., Goh, V. H. H., Ridgway, E. C. & Wierman, M. E. (2011). Re-examination of testosterone, dihydrotestosterone, estradiol and estrone level across the menstrual cycle and in postmenopausal women measured by liquid chromatography-tandem mass spectrometry. *Steroids*, 76: 177 - 182.

Sato, K., Iemitsu, M., Aizawa, K. & Ajisaka, R. (2008). Testosterone and DHEA activate the glucose metabolism-related signalling pathway in skeletal muscle. *American Journal of Physiology, Endocrinology & Metabolism,* 294: E961–E968.

Schwarz, A. J., Brasel, J. A., Hintz, R. L., Mohan, S. & Cooper, D. M. (1996). Acute effect of brief low- and high-intensity exercise on circulating insulin-like growth factor (igf) i, ii, and igf-binding protein-3 and its proteolysis in young healthy men. *Journal of Clinical Endocrinology and Metabolism,* 81: 3492-7.

Schwartz, E.B., Eppihimer, L., Nelson, V.J. & Granger, D.A. (2005). Effects of sample storage temperature on the measurement of salivary analytes: Degradation of testosterone, estradiol, and progesterone. *Clinical Chemistry,* 51: A48-A49.

Scott, J. P., Sale, C., Greeves, J. P., Casey, A., Dutton, J. & Fraser, W. D. (2011). Effect of exercise intensity in the cytokine response to an acute bout of running, *Medicine and Science in Sports and Exercise,* e-publication before print.

Smith, A. A., Toone, R., Peacock, O., Drawer, S., Stokes, K. A. & Cook, C. J. (2013). Dihydrotestosterone is elevated following sprint exercise in healthy young men. *Journal of Applied Physiology*, 114: 1435-1440.

Smith, M. D., Jones L.S. & Wilson, M. A. (2002). Sex differences in hippocampal slice excitability: role of testosterone. *Neuroscience*, 109 (3): 517-30

Spiering, B. A., Kraemer, W. J., Anderson, J. M., Armstrong, L. E., Nindl, B. C., Volek, J. S. & Maresh, C. M. (2008). Effects of elevated circulating hormones on resistance exercise-induced Akt signaling. *Medicine and Science in Sports and Exercise,* 40(6): 1039-1048.

Spiering, B. A., Kraemer, W. J., Anderson, J. M., Armstrong, L. E., Nindl, B. C., Volek, J. S., Maresh, C. M. (2008). Resistance exercise biology: manipulation of resistance exercise programme variables determines the responses of cellular and molecular signalling pathways. *Sports Medicine,* 38: 527–540.

Stokes, K. A., Gilbert, K. L., Hall, G. M., Andrews R. C. and Thompson, D. (2013). Different responses of selected hormones to three types of exercise in young men. *European Journal of Applied Physiology*, 113: 775-783.

Stokes, K., Nevill, M., Frystyk, J., Lakomy, H. & G. Hall. (2005). Human growth hormone responses to repeated bouts of sprint exercise with different recovery periods between bouts, *Journal of Applied Physiology,* 99: 1254-1261.

Stokes, K. A., Sykes, D., Gilbert, K., Chen, J. W. & Frystyk, J. (2010). Brief, high intensity exercise alters serum ghrelin and growth hormone concentrations but not IGF-I, IGF-II or IGF-I bioactivity, *Growth Hormone & IGF Research,* 20: 289 – 294.

Stone, M. R., Thomas, K., Wilkinson, M., St Clair-Gibson, A. & Thompson, K. G. (2011). Consistency of perceptual and metabolic responses to a laboratory-based simulated 4,000-m cycling time trial. *European Journal of Applied Physiology*, 111(8): 1807-1813.

Suzuki, Y., Doukyu, N. & Aono, R. (1998). Lithocholic acid side-chain cleavage to produce 17-keto or 22-aldehyde steroids by pseudomonas putida strain st-491 grown in the presence of an organic solvent, diphenyl ether. *Bioscience, Biotechnology & Biochemistry,* 62: 2182-8.

Taipale, R. S. & Hakkinen, K. (2013). Acute hormonal and force responses to combined strength and endurance loading in men and women: the 'order effect'. *PLoS ONE*, 8(2): e55051.

Taylor, L., Fitch, N., Castle, P., Watkins, S., Aldous, J., Sculthorpe, N., Midgely, A., Brewer, J. & Mauger, A. (2014). Exposure to hot and cold environmental conditions does not affect the decision making ability of soccer referees following an intermittent sprint protocol. *Frontiers in Physiology*, 5: 1-9.

Thigpen, A. E., Silver, R. I., Guileyardo, J. M., Casey, M. L., McConnell, J. D. & Russell, D. W. (1993). Tissue distribution and ontogeny of steroid 5 alpha-reductase isozyme expression. *Journal of Clinical Investigation,* 92: 903–910.

Thomas, K., Stone, M. R., Thompson, K. G., St Clair Gibson, A. & Ansley, L. (2012). Reproducibility of pacing strategy during simulated 20-km cycling time trials in well-trained cyclists. *European Journal of Applied Physiology*, 112: 223-229.

Tillin, N. A. & Bishop, D. (2009). Factors Modulating Post-Activation Potentiation and its Effect on Performance of Subsequent Explosive Activities. *Sports Medicine*, 39 (2): 147-166

Tremblay, M. S., Copeland, J. L. & Van Helder, W. (2004). Effect of training status and exercise mode on endogenous steroid hormones in men. *Journal of Applied Physiology,* 96: 531-9.

Tremblay, M. S., Copeland, J. L. & Van Helder, W. (2005). Influence of exercise duration on post-exercise steroid hormone responses in trained males. *European Journal of Applied Physiology,* 94: 505-13.

Urhausen, A., Gabriel, H. & Kindermann, W. (1995). Blood hormones as markers of training stress and overtraining. *Sports Medicine,* 20: 251-76.

VanBruggen, M. D., Hackney, A. C., Murray, R. G. & Ondrak, K. S. (2001). The relationship between serum and salivary cortisol levels in response to different intensities of exercise. *International Journal of Sports Physiology and Performance,* 6(3): 396-407.

Vining, R. F., McGinley, R. A., & Symons, R. G. (1983). Hormones in saliva: mode of entry and consequent implications for clinical interpretation. *Clinical Chemistry, 29*(10): 1752-1756.

Viru, A.M., Hackney, A.C, Valja, E., Karelson, K., Janson, T. & Viru, M. (2001). Influence of prolonged continuous exercise on hormone responses to subsequent exercise in humans. *European Journal of Applied Physiology*, 85: 578-585.

Volek, J. S. (2004). Influence of nutrition on responses to resistance training. *Medicine and Science in Sports and Exercise,* 36(4): 689-696.

Volek, J. S., Kraemer, W. J., Bush, J. A., Incledon, T., & Boetes, M. (1997). Testosterone and cortisol in relationship to dietary nutrients and resistance exercise. *Journal of Applied Physiology,* 82(1): 49-54.

West, D. W., Burd, N. A., Tang, J. E., Moore, D. R., Staples, A. W., Holwerda, A. M., Baker, S. K. & Phillips, S. M. (2010). Elevations in ostensibly anabolic hormones with resistance exercise enhance neither training-induced muscle hypertrophy nor strength of the elbow flexors. *Journal of Applied Physiology,* 108(1): 60-67.

West, D. W. D & Phillips, S. M. (2011). Associations of exercise-induced hormone profiles and gains in strength and hypertrophy in a large cohort after weight training. *European Journal of Applied Physiology,* 95: 421-426.

Whembolua, G. L., Granger, D. A., Singer, S., Kivlighan, K. T. & Marguin, J. A. (2006). Bacteria in the oral mucosa and its effects on the measurement of cortisol,

dehydroepiandrosterone, and testosterone in saliva. *Hormones & Behaviour,* 49: 478-83.

White, L.. J., Dressendorfer, R. H., Ferguson, M. A. & Wade, C. E. (2002). Maintenance of testosterone status in fitness joggers after increased training mileage. *European Journal of Applied Physiology*, 86: 498 - 502.

Wilkinson, S. B., Tarnopolsky, M. A., Grant, E. J., Correia, C. E., & Phillips, S. M. (2006). Hypertrophy with unilateral resistance exercise occurs without increases in endogenous anabolic hormone concentration. *European Journal of Applied Physiology,* 98(6): 546-555.

Wilson, J. M., Duncan, N. M., Marin, P. J., Brown, L. E., Loenneke, J. P., Wilson, S. M. C., Jo, E., Lowery, R. P. & Ugrinowitsch, C. (2013). Metaanalysis of postactivation potentiation and power: Effects of conditioning activity, volume, gender, rest periods, and training status. *Journal of Strength and Conditioning Research*, 27(3): 854–859.

Wood, P. (2009). Salivary steroid assays - research or routine? *Annals of Clinical Biochemistry,* 46: 183-96.

Yoshioka, M., Boivin, A., Ye, P., Labrie, F. & St-Amand J. (2006). Effects of dihydrotestosterone on skeletal muscle transcriptome in mice measured by serial analysis of gene expression. *Journal of Molecular Endocrinology,* 36: 247–259.

Zaki, A. & Barrett-Jolley, R. (2002). Rapid neuromodulation by cortisol in the rat paraventricular nucleus: an in vitro study. *British Journal of Pharmacology,* 137 (1): 87-97

Zanker, C. L & Swaine, I. L. (2000). Responses of bone turnover markers to repeated endurance running in humans under conditions of energy balance or energy restriction, *European Journal of Applied Physiology,* 83: 434 – 440.

Zimmerman, Y., Eijkemans, M. J. C., Coelingh Bennink, H. J. T., Blankenstein, M. A. & Fauser, B. C. J. M. (2014). The effect of oral contraception on testosterone levels in healthy women: a systematic review and meta-analysis. *Human Reproduction Update*, 20(1): 76 - 105.