The Relationship between Plasma and Salivary Cortisol Levels in Response to Different Exercise Intensities

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ABSTRACT

MITCH D. VANBRUGGEN: The Relationship between Plasma and Salivary Cortisol Levels in Response to Different Exercise Intensities
(Under the direction of Anthony C. Hackney, Ph.D., D.Sc.)

This study examined the effect of exercise intensity on the serum and salivary cortisol responses of endurance-trained males. Subjects (n = 12) rested for 30 minutes (control) and exercised for 30 minutes at 40%, 60%, or 80% of $VO_{2\ max}$ on separate days. Serum and saliva samples were collected pre-trial, post-trial, and 30 minutes post-recovery. The overall correlation between serum and saliva in all matched pairs was significant ($r = 0.548$; $p < 0.005$). Cortisol responses increased significantly with both measures in response to exercise ($p < 0.05$). However, exercise peak serum responses occurred at the post-trial time while saliva peaked at the post-recovery time. The highest correlations between serum-saliva at individual sampling times were during post-recovery. Findings suggest that salivary cortisol sampling may be a useful technique in some circumstances if confounding factors are considered and controlled.

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CHAPTER I

BASIS FOR STUDY

Introduction

Cortisol levels increase in response to psychological and physical stressors such as life changes, extreme temperatures, negative energy balance, and physical exercise (McMurray & Hackney, 2000; Viru et al., 2004). In response to the stress of exercise, cortisol has many specific functions helping the body modify and adapt to the stress, including: the mobilization of free fatty acids (FFA) from adipose tissue, protein catabolism, stimulation of gluconeogenesis at the liver, and inhibition of glucose uptake by the working skeletal muscle (Brooks et al., 2000). These responses act to increase exercise capacity and aid in recovery and adaptation (Viru et al., 2007).

When stressed, the hypothalamus secretes corticotrophin releasing hormone (CRH), which activates the anterior pituitary and stimulates the release of adrenocorticotropin hormone (ACTH). The presence of ACTH stimulates the adrenal cortex to release cortisol (Neal, 2001). Cortisol secretion is controlled through a negative feedback process, where high levels inhibit the secretion of ACTH from the anterior pituitary. In contrast, high levels of ACTH and cortisol can signal the hypothalamus to reduce the secretion of CRH. This entire interconnected process is referred to as the hypothalamic-pituitary-adrenocortical (HPA) axis (Hill et al., 2008).

Most previous exercise studies investing cortisol responses to exercise are in agreement that there is a "threshold intensity" that results in significant elevations in circulating cortisol. For example, Davies and colleagues (1973) found that an exercise intensity of 50-60% of VO_{2max} must be reached for cortisol to be increased and the absolute levels attained during exercise are dependent on the total duration of the exercise bout. In another example, Hill and colleagues (2008) examined the effect of exercise intensity upon the cortisol response of the HPA axis in moderately trained men. Moderate to high intensity (60% and 80% of maximal oxygen consumption [$VO_{2\ max}$]) exercise augmented circulating cortisol levels. These increases were a result of a combination of hemoconcentration and HPA axis stimulus (ACTH). Conversely, low intensity exercise (40% of $VO_{2\ max}$) did not result in significant increases in cortisol levels, actually reducing cortisol concentrations when accounting for plasma volume changes (Hill et al., 2008).

While there are similar findings in previous research assessing cortisol release and circulation during exercise, many of these studies measured the cortisol response by assessing concentrations in the serum of the blood. While this method is accurate and consistent, collection of blood samples is invasive, difficult to obtain during the exercise process, and can also augment the stress response of the subject due to the collection procedure (Kirschbaum et al., 1994).

Cortisol concentrations measured in saliva may provide a feasible, accurate, and practical alternative to serum determinations. In fact, some laboratories, clinicians, and neuroendocrine researchers already use this technique (Hellhammer et al., 2009). Since cortisol is a lipophilic steroid with low molecular weight, the cortisol that is unbound to

carrier proteins (e.g. cortisol binding globulin [CBG]) can enter the cells through passive diffusion. In this way, it is possible to measure these free cortisol levels in all bodily fluids, including saliva. Since only a small, unbound fraction of the hormone is available to diffuse into the saliva, concentrations are consistently lower than in serum. However, it has been demonstrated that salivary cortisol levels have a steady and predictable relation to the free, unbound cortisol levels in serum and salivary levels accurately reflect serum levels regardless of the degree of stimulation of the saliva glands (Vining et al., 1983). Previous studies have found correlation coefficients between cortisol in saliva and cortisol in serum ranging from $r = 0.71$ to $r = 0.96$ (Kirschbaum et al., 1994). However, these cross-sectional correlations reflect associations between the two methods when subjects were at rest—exercise studies were not included in the review.

Though many studies consider salivary cortisol concentrations a reliable and accurate measure of this hormone, there is still some controversy surrounding this technique. Due to the passive movement of cortisol from the serum into the saliva, there may be a delayed response in salivary concentrations accurately reflecting the response in the blood (Umeda et al., 1981). Additionally, because the response of the HPA axis is controlled through many processes and factors, cortisol levels in the saliva may be partly disassociated from the other processes regulating the HPA feedback loop, including ACTH and CRH. Moreover, previous studies comparing methodologies have not used exercise as the stressor to stimulate the HPA axis (Hellhammer et al., 2009). The acute stress of short exercise bouts (compared to prolonged exercise) may not allow for sufficient time for cortisol to diffuse into saliva. Thus, this methodology needed to be further explored, particularly in response to extreme stressors, such as physical exercise.

Purpose

This purpose of this study was to investigate the associations between serum and salivary cortisol levels pre-trial, immediate post-trial and after recovery (30 minutes; post-recovery) at three different intensities (40%, 60%, and 80% of $VO_{2\ max}$) and a control trial to determine if salivary concentrations could be an accurate method to assess the body's stress response to exercise. It also aimed to determine if there was a similar temporal relationship for peak responses to exercise.

Research hypotheses

1. There will be significant correlations between serum and salivary cortisol concentrations at the pre-trial, post-trial and post-recovery sampling time within each of the experimental trials (control, 40%, 60% and 80% $VO_{2\ max}$ exercise).

2. The peak saliva cortisol response when compared to the peak serum response within the 60% and 80% exercise trials will occur at a later sampling time.

Definition of terms

Adrenocorticotropic hormone (ACTH) –a polypeptide tropic hormone produced and secreted by the anterior pituitary gland (Neal, 2001).

Cortisol – A glucocorticoid hormone secreted from the zona fasciculata of the adrenal cortex. Cortisol release is stimulated by adrenocorticotropin hormone (ACTH) from the anterior pituitary (Neal, 2001).

Cortisol binding globulin (CBG) –a large plasma protein that the majority of cortisol is bound to as it travels in the blood (Kirschbaum et al., 1994).

Corticotropin releasing hormone (CRH) – a hormone secreted by the paraventricular nucleus (PVN) of the hypothalmus in response to stress (Neal, 2001).

Hypothalamic-pituitary-adrenocortical (HPA) axis – an interconnected feedback loop which is a major part of the neuroendocrine system that controls reactions to stress and regulates many of the body's processes.

Maximal oxygen uptake ($VO_{2\ max}$) – the maximum capacity to transport and utilize oxygen during whole-body exercise (Brooks et al., 2000).

Radioimmunoassay (RIA) – a highly sensitive laboratory technique that analyzes of the binding between an antigen and its homologous antibody in order to identify and quantify a substance (e.g. hormone) in a biological fluid (Chard, 1990).

Salivary cortisol enzyme immunoassay (EIA) – a highly sensitive immunochemical test in which cortisol in standards and unknowns compete with cortisol linked to horseradish peroxidase for the antibody binding sites. The amount of cortisol peroxidase detected is inversely proportional to the amount of cortisol present (Chard, 1990).

Delimitations

1. Subjects were healthy, endurance-trained males between 18 and 30 years of age.
2. Subjects reported to each trial 4 hours post-prandial, and maintained and controlled their diet preceding each of the experimental trials.
3. Experimental trials were completed in random order, separated by at least 72 hours.
4. Each of the experimental trials were conducted at the same time of day (within each subject) to account for circadian rhythms.

5. Psychological stress was controlled for as each subject demonstrated normal scores on the Recovery-Stress Questionnaire (REST-Q) before proceeding with each of the separate experimental trials.

Limitations

1. The results can only be generalized to the sample studied: healthy, moderately to highly endurance trained adult males between 18 and 30 years of age.
2. The sample size in this study was relatively small (n = 12) due to limitations in recruiting moderate to highly trained males.

Significance of study

This study attempted to validate salivary cortisol as a reliable method to assess levels of this hormone in response to exercise. Collection of saliva (as opposed to blood) facilities more frequent sampling, is less invasive, and allows subjects to obtain samples without the assistance of a phlebotomy technician. This permits sampling outside of a laboratory environment such as during a training session or competitive event. This study is novel because it involved assessing each of these methods in response to different exercise intensities and determined if there was a delay in the peak responses of saliva compared to serum, which has not been extensively studied in previous research. If this relationship between these two methods is better understood, it may facilitate saliva sampling as a more readily acceptable measurement in sports physiology.

CHAPTER II

REVIEW OF LITERATURE

Introduction

It is important to note that there are few previous research studies which have specifically used salivary cortisol sampling techniques, especially in response to exercise protocols utilizing multiple intensities and in comparison to serum sampling techniques. This makes the purpose of this research study somewhat novel; however, it was also a limitation in that that there are few studies which provided an extensive groundwork and foundation to build upon and compare to in this project.

Physiological mechanisms of cortisol release

Theory and overview

Cortisol secretion is controlled through the mechanisms of the hypothalamus, pituitary gland, and adrenal cortex (HPA axis). In response to physiological and psychological stressors, the hypothalamus secretes corticotrophin-releasing hormone (CRH) which causes the anterior pituitary gland to secrete adrenocorticotropin releasing hormone (ACTH) into circulation. This hormone then causes the adrenal cortex to release cortisol. As the cortisol level is augmented, CRH and ACTH are inhibited through negative feedback (Brooks et al., 2000; McMurray & Hackney, 2000).

Cortisol release is stimulated by a wide array of stress-inducing stimuli, including exercise, which results in a wide array of mechanisms which help the body adapt to the stress

and return to homeostasis (Neal, 2001). The primary roles of cortisol include: proteolysis, stimulation of lipolysis, and promoting gluconeogenesis at the liver (Hackney, 2006; McMurray & Hackney, 2000). Cortisol also prevents the uptake of glucose by the active skeletal muscle and has an inhibitory affect on protein synthesis during exercise and into the recovery period following an exercise bout. Inhibition of protein synthesis in recovery functions to free amino acids into the blood which are used for muscle remodeling, to build new proteins, or to be shunted to the hepatic tissue to be used for substrate in gluconeogenesis. This is why cortisol levels often remain elevated for up to 120 minutes into recovery from the exercise bout (Viru et al., 2004). Figure 1 displays the control of the HPA axis and the effect of cortisol on the body's metabolic processes.

Factors influencing variability of responses

Acute exercise can result in increased cortisol levels, but the response of the hormone is largely dependent on the intensity of the exercise bout being completed. The cortisol response during an acute exercise session is dependent on many other factors, including: circadian rhythms, environmental conditions, competitive nature of exercise bout, age, gender, genetics, anaerobiosis of the exercise, and nutritional considerations (Hackney, 2006; Thuma et al., 1995).

Training status is also a key component in cortisol responses- levels in trained subjects are generally more attenuated than those who are untrained or sedentary, even when the physically fit subjects work at the same percentage of their maximal capacity as the unfit subjects. Additionally, trained athletes typically have lesser responses as the duration of the exercise becomes longer and the effort becomes more difficult (Bloom et al., 1976). This may be due to a higher number of receptors as well as an increased sensitivity to cortisol,

meaning that less of the hormone is needed to produce the responses required to adapt to the stress of the exercise (Powers & Howley, 2004).

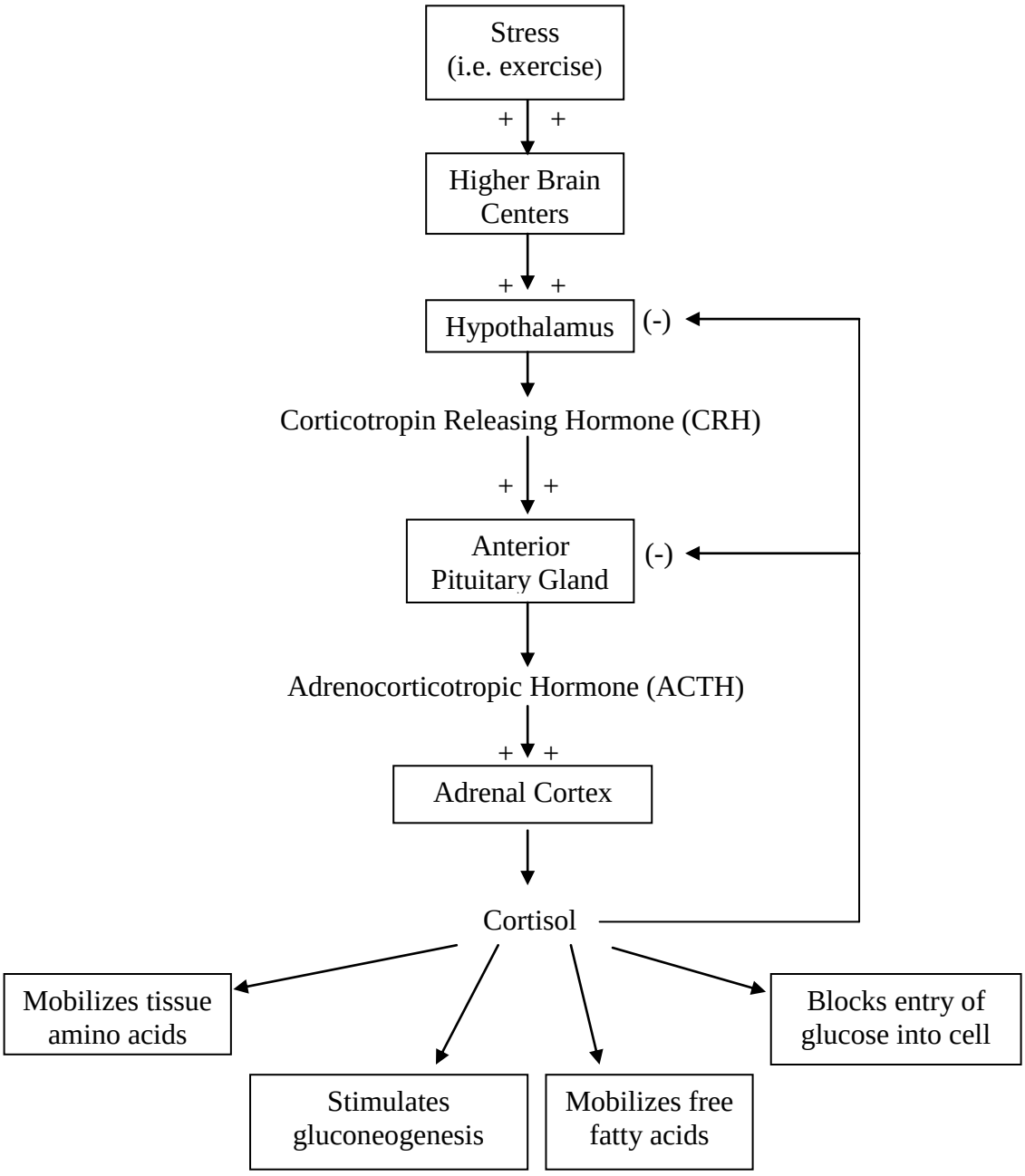

Figure 1. Control of cortisol release in the hypothalamic-pituitary-adrenal (HPA) axis, highlighting the positive and negative feedback to the hypothalamus and cortisol's effects on the body's metabolic processes (adapted from Powers & Howley, 2004).

Serum cortisol sampling with exercise

Previous research studies which have analyzed subjects' cortisol responses to exercise have primarily utilized serum sampling to assess this hormone. Daly and colleagues examined the cortisol responses to exhausting, prolonged exercise. Thirty-four healthy male subjects ran on a treadmill until volitional exhaustion. Blood specimens were analyzed for cortisol levels immediately at the end of exercise and at 30, 60, and 90 minutes into recovery. A significantly greater number of the peak cortisol responses occurred during the recovery period, indicating the importance of analyzing the cortisol responses into the recovery period and not simply before, during or immediately after the exercise session. (Daly et al., 2004).

Viru and colleagues also utilized serum sampling to assess cortisol responses to maximal exercise and how this was affected by adrenergic factors. They had ten subjects run to exhaustion on a treadmill, and obtained blood samples pre-exercise and post exercise to be used to determine how the cortisol responses were affected by beta-adrenergic blockage as well as a competitive condition. They found that both of these factors significantly augmented cortisol responses ($n = 10$; $p < 0.05$), but were not additive, suggesting that that there may be an upper limit on the magnitude of the of HPA response to intense exercise (Viru et al., 2007).

Davies and Few (1973) investigated the cortisol response of ten subjects to a light load (<50% VO_{2max}) and a heavy load (60-90% VO_{2max}). They determined that there needed to be a threshold intensity of approximately 60% VO_{2max} in order for cortisol levels to increase. Hill and colleagues (2008) also verified that a workload of at least 60% VO_{2max} will

augment cortisol responses. In this study, twelve moderately trained males exercised at 40%, 60%, and 80% of VO_{2max}. Both the 60% and 80% trials resulted in significantly higher cortisol responses than during the resting (control) and 40% trial. In fact, during the 40% exercise bout, there was a decrease in cortisol (Hill et al., 2008). This is consistent with the explanation of Galbo (1983). Cortisol responses may appear to be decreasing in a subjects' serum, but this is just a turnover effect. Cortisol is being released during the low intensity exercise, but the clearance rate is simply greater than what is being secreted, resulting in a lower concentration in the serum (Galbo, 1983). Other studies cited in review articles and textbook chapters have corroborated the intensity/threshold effect of cortisol, noting that approximately 60% of maximal aerobic capacity must be reached in order for cortisol levels to respond to the exercise (Brooks et al., 2000; McMurray & Hackney, 2000).

Salivary cortisol methodology

Diffusive properties, sampling, and analysis

Control over saliva production is shared by the sympathetic and parasympathetic branches of the autonomic nervous system, which work together in an intricate, complex relationship. The parasympathetic system is largely responsible for enhancing fluid secretion by the salivary glands, with the sympathetic system playing a smaller role. However, both of these systems can signal the myoepithelial cells in the salivary glands to contract, increasing the flow of saliva (Garrett, 1987).

Some compounds in the body can pass into the saliva from the blood, making saliva a viable and safe diagnostic fluid in many areas of scientific research, especially when compared to blood and urine (Kaufman et al., 2002). Specifically, cortisol has a steady and

predictable relation to the free, unbound levels in serum. Salivary cortisol is a useful biomarker in stress research, as long as the researcher is aware of possible sources of variation, which may affect this measure. Several factors, including adrenal sensitivity and cortisol binding affect total and free cortisol levels in blood, translating to what concentrations are measured in the saliva (Hellhammer et al., 2009).

In a series of studies, Schwartz and colleagues evaluated the reliability of radioimmunoassay (RIA) for salivary cortisol. They concluded that associations between serum and salivary cortisol were reliable and valid as long as contaminants were not introduced into the sampling process (Schwartz et al., 1998). Umeda and colleagues (1981) also examined the viability of salivary cortisol measurement, stating that it is an exceptionally accurate index of plasma free cortisol concentration, independently of salivary flow rate. Additionally, Umeda maintained that this method may be preferable to other methods for many reasons: saliva is obtained by noninvasive stress-free procedures, is stable at room temperatures, is easier to collect, does not require skilled personnel for collection, and can be sample numerous times during a session in the laboratory or throughout the day at home or in the field (Levine et al., 2007).

Salivary cortisol sampling with exercise

Kivlighan and colleagues used saliva as the sampling method for obtaining cortisol responses to the anticipation of exercise as well as during the recovery period after the exercise. Though this study did not specifically focus on the methodology of the saliva sampling technique, it was unique in that the cortisol responses of the subjects were highly consistent with previous responses measured using serum concentrations. That is, the

responses to the particular protocol of exercise and recovery mirrored cortisol concentrations measured via serum (Kivlighan et al., 2005). However, this protocol utilized only one bout of exercise (2000 m rowing ergometer sprint) and did not measure responses to various exercise intensities, thus resulting in only one level of stress.

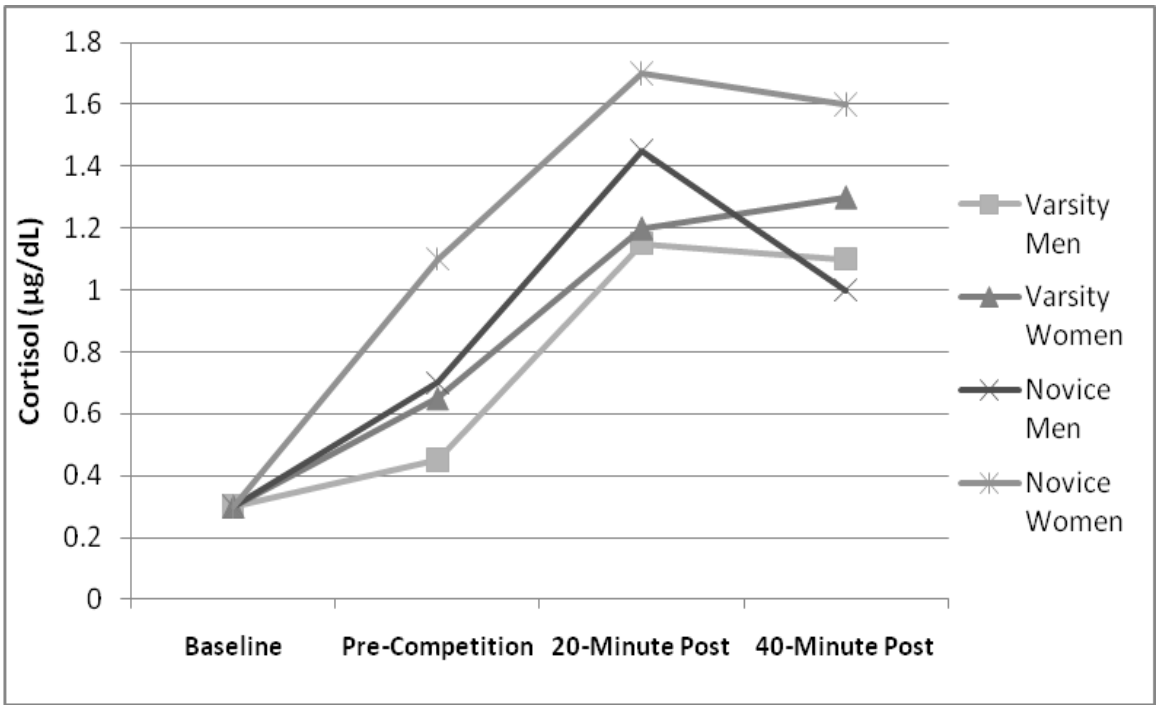

Figure 2. Saliva cortisol responses in trained and untrained men and women before competition, 20 minutes into recovery, and 40 minutes into recovery from the competitive exercise bout (adapted from Kivlighan et al., 2005).

Jacks and colleagues also used saliva as a sampling technique in a study involving exercise and cortisol responses—however, this study measured responses to differences in exercise intensities. Ten males were assigned to random, 1 hour-cycle ergometer bouts of exercise at approximately 40%, 60%, and 80% of their VO_2 peak as well as a resting control session. The saliva samples were collected before exercise and then at 10, 20, 40, and 59 minutes of exercise and at 20 minutes of recovery. They found that with the 80% exercise

intensity, cortisol was significantly higher at 59 minutes of exercise than at those same time points during the resting control session. No significant differences in cortisol concentration were found in the other exercise intensities (Jacks et al., 2002). This study was very similar in methodology to the present study, but utilized salivary sampling exclusively to determine the subjects' response to different exercise intensities and did not compare serum and sampling techniques and concentrations.

Comparison of serum and saliva sampling

Previous studies have found strong relationships between the two sampling methods, evidenced by strong correlation coefficients between cortisol in saliva and cortisol in serum at rest ranging from $r = 0.71$ to $r = 0.96$ (Kirschbaum et al., 1994). For instance, Vining and colleagues found that salivary cortisol concentrations were directly proportional ($p < 0.05$) to the serum unbound cortisol concentration both in normal men and women and that the rate of equilibrium of cortisol between blood and saliva was very fast (less than 5 minutes). However, this investigation did not use exercise as the stressor to the HPA axis, which may have caused a slower equilibrium between free levels of the hormone in blood and saliva. Still, the researchers concluded that salivary cortisol is the more appropriate measure of the response of the HPA axis to stress than serum cortisol since it is more simple, stress free, and non-invasive (Vining et al., 1983). Associations between the methods also remained high ($r = 0.86$; $p < 0.05$) when measuring cortisol with both methods throughout the circadian cycle of the hormone (Levine et al., 2007)

Comparisons of methods in response to exercise

Few studies have directly compared the two sampling techniques in response to exercise. Ben-Aryeh and colleagues examined the effect of exercise on cortisol levels in serum and saliva in young, healthy males. The subjects performed graded submaximal cycle exercise for nine min at up to 85% of their age-predicted maximal heart rate. Surprisingly, they found a non-significant increase in saliva and serum cortisol levels; however, the lack of significance was attributed to a decrease in blood flow in the salivary glands, dry mouth due to dehydration, and a decrease in measured salivary flow. They also postulated that collection of the saliva samples later on during the recovery period would have yielded significant increases in cortisol concentrations as opposed to what was found immediately after the exercise session (Ben-Aryeh et al., 1989). This study highlighted the importance of the timing of the serum and saliva sampling to limit other confounding variables that can affect the results of the cortisol responses in each of these fluids.

Thomasson and colleagues directly compared plasma and saliva hormones in response to exercise; however, the investigation focused on the hormones' response to a long exercise session (120 minutes) rather than a shorter, more intense bout. Nine, healthy subjects exercised for the 120 minutes period at 50-55% of their VO_{2max}. Blood and saliva samples were taken at rest and every 30 minutes during the exercise bout and found there was a significant relationship ($r = 0.35$; $p < 0.02$) between the concentrations of cortisol in each of these biological fluids averaged over the exercise sessions. They concluded that the non-invasive saliva sampling offers a realistic and practical approach in the measurement of

the response of the HPA axis to exercise and can be used as an alternative to serum sampling (Thomasson et al., 2009).

Research involving comparisons between serum and saliva using high intensity exercise have yielded mixed results immediately after exercise. Gonzansky and colleagues designed a study to determine whether salivary cortisol could be used instead of serum cortisol in across a broad range of concentrations. They found that the salivary cortisol responses to brief, intense exercise (90% of maximal heart rate for 10 minutes) paralleled serum cortisol ($r = 0.60$; $p < 0.001$). The authors asserted that the salivary measures actually are advantageous compared to serum during intense exercise because serum concentrations are affected by the saturation point of cortisol binding globulin (CBG). Therefore, using salivary cortisol as opposed to total serum cortisol eliminates the requirement to account for within-subject changes or between-subject differences in CBG (Gonzansky et al., 2005). Stupnicki and Obminski assessed serum and salivary cortisol concentrations in 78 elite athletes engaged in different sports, by subjecting them to high-intensity laboratory exercise. The mean difference in the pre-exercise cortisol concentrations in the seven groups studied were more marked in serum than in saliva. The correlations between the pre-exercise values were 0.47 for serum and 0.58 for saliva. This led the researchers to suggest that the salivary cortisol concentration might be a more suitable variable for assessing glucocorticoid activity before exercise and in response to exercise compared to serum cortisol concentrations since it is likely less sensitive to pre-exercise emotional state (Stupnicki & Obminski, 2002).

While concentrations measured immediately after exercise are useful when making comparisons between serum and saliva, there is limited research assessing responses into recovery from exercise. Neary and colleagues investigated the relationship among resting

cortisol levels measured in serum and saliva samples to determine which method would be the most appropriate and preferable to observe and monitor the physiological stress of exercise training. Serum and saliva samples were collected from eight subjects following one day of recovery from intense training. They found a significantly high correlation between serum and salivary cortisol ($r = 0.99$) and concluded that either sampling technique can be used to monitor cortisol during a recovery period from exercise training. However, since the saliva collection is much less invasive, they maintained that it was the preferable method (Neary et al., 2002). Finally, del Corral and colleagues assessed serum and salivary cortisol responses of young males during and after a 70% exercise session. There were significant increases in cortisol responses to the exercise session with both methods. Additionally, the two methods were correlated ($p < 0.05$) after 30minutes of exercise ($r = 0.90$), and 15 min post-exercise ($r = 0.84$). However, the levels were not correlated at rest ($r = 0.46$). This is one of the few studies which used both methods and assessed levels during recovery from exercise rather than simply immediately after exercise (del Corral et al., 1994). However, responses were not assessed using multiple intensities as is design of the present study.

Need for additional research on salivary cortisol in response to exercise

This study aimed to validate salivary cortisol sampling as a way to assess this stress related hormone in response to exercise intensity. While some previous studies have investigated salivary cortisol response to exercise, there is minimal research which has attempted to validate salivary cortisol responses to exercise using multiple intensities. The goal and unique aspect of this study was to examine if there is still a similar response in salivary concentrations to the established response in serum concentrations. With multiple

exercise intensities, it was necessary to investigate whether the relationships between these two methods are disassociated from one another, or if there was a mirrored relationship when comparing responses in serum and saliva. Determining when the peak responses of cortisol occur in response to different exercise intensities using both methods may significantly contribute to the field of sports physiology. Since salivary methods are more practical and less invasive, future research may be possible in field settings, during training sessions, or before and after a competitive event.

CHAPTER III

METHODOLOGY

Subjects

Moderate to highly aerobically-trained male subjects (ages 18-30) were recruited from the campus of the University of North Carolina-Chapel Hill and surrounding areas for this study. Subjects must have trained consistently for a minimum of 3 days per week for 60 minutes per day in the previous six months prior to the study. Each of the subjects were informed of the risks of the protocol, signed statement of informed consent, passed a medical and physical examination, and demonstrated normal scores on the Recovery Stress Questionnaire (REST-Q) prior to each experimental trial. If the subjects scored above the average of the midpoints of the stress scales, they were not allowed to proceed on that particular trial. Exclusion criteria included: a diet chronically low (<50% of daily caloric consumption) in carbohydrates, a prior history of hormonal disorders, mental illness, or chronic non-steroidal anti-inflammatory (NSAID) drug use.

Protocol

Each subject was asked to report to the Applied Physiology Laboratory at the University of North Carolina-Chapel Hill on five separate occasions. Subjects were instructed to maintain and control their diet (eucaloric and at least 50% of calories from carbohydrates) over the duration of the study. Prior to when the subjects reported to the laboratory for the orientation session, they completed a 3-day diet record which they brought

with them to the laboratory. In between the orientation session and the first experimental trial, the diet was analyzed using the nutrition database on the website of the United States Department of Agriculture to determine the macronutrient breakdown of their daily caloric intake. If the subjects' diet did not meet the required average daily carbohydrate consumption to proceed, they were given guidance on how to healthily incorporate additional sources of this macronutrient into their diet. After they completed another 3 day diet record and verified that their diet contained at least 50% of calories from carbohydrates, the subjects were allowed to continue with the experimental trials. Furthermore, subjects were asked to come to laboratory 4 hours post-prandial, having consumed no caffeine or alcohol for 8 hours prior to the session.

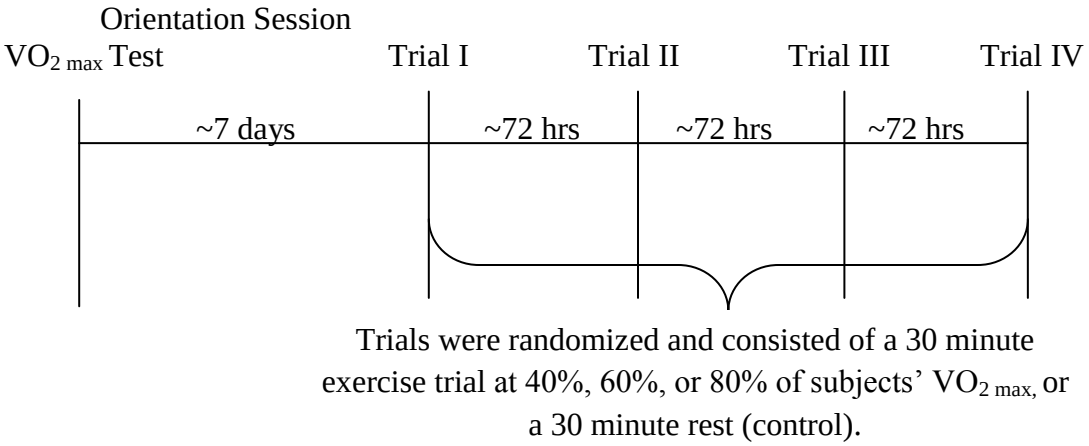

Figure 3. Overview of experimental protocol.

The first session served as both an orientation session and when the subjects' maximal oxygen uptake ($VO_{2\ max}$) was determined through an incremental exercise test on a cycle ergometer using 3 minute stages. Following the initial meeting and $VO_{2\ max}$ test, the next four experimental trials consisted of a control trial, and 30 minute cycling bouts at 40%,

60%, and 80% of the subjects' $VO_{2\ max.}$ All of these trials were completed at the same time of day within each subjects' trials (± 30 minutes), were in a randomized order, and each trial was separated by a minimum of 72 hours.

Orientation/maximal oxygen consumption ($VO_{2\ max}$) testing session

Subjects were instructed to refrain from any physical exercise for 24 hours before their $VO_{2\ max}$ test. Once subjects came to the laboratory, they were briefed on the protocol (exercise, blood collection procedures, metabolic monitoring) and were allowed to ask any questions about the procedure before signing the informed consent form. Next, the subjects underwent a screening process, including a blood pressure reading, a 12-lead electrocardiogram, and a brief discussion on previous illnesses and family history of potential problems to ensure that they could safely participate in the study. After approval for participation, anthropometric data (age, height, weight, body fat percentage via skinfolds) were determined for each subject.

After subject characteristics were assessed, they were instructed to warm-up on the cycle ergometer for 5 minutes at a very light workload. At this time, the seat height was adjusted to the optimal height and comfort. The light warm up was followed up by 5 minutes of stretching, primarily emphasizing the torso and the lower extremities. The subject was subsequently properly fit with a mouthpiece and oxygen uptake which was used to make sure that the values were normal and the metabolic system was functioning properly.

The incremental exercise test began at a workload previously determined by the subjects' training history and any previous $VO_{2\ max}$ data results reported by the subject. The workload increased at the end of the 3 minute intervals (stages) until volitional fatigue.

Metabolic data was averaged over 15 second intervals, heart rate data was recorded every minute, and ratings of perceived exertion (RPE) were assessed at the end of each exercise stage. The test was considered valid and reliable if the subjects met three out of the four criteria: a 150 ml/min or less increase in VO_2 in response to an increased workload; HR at the age predicted maximum (within 5%); a respiratory exchange ratio (RER) of 1.1 or greater, and an RPE rating of 18 or greater (ACSM, 2000).

Exercise-control trials

Subjects reported to the laboratory on a separate day, at least 72 hours but no more than 7 days following their initial visit. They reported to the laboratory for these tests at the same time of day (±30 minutes) for each trial. During these testing trials, the subjects had three blood draws performed and concurrently three saliva samples collected.

First, the subjects filled out the REST-Q questionnaire and if normal scores were demonstrated, they began their supine resting period. After 30 minutes, pre-exercise blood and saliva samples were taken. Next, the subject began a 5-minute warm up on the cycle ergometer at very low intensity (approximately 15-20 % of $VO_{2\ max}$). Subjects were then instructed to stretch for 5 minutes after which they mounted the cycle and began the exercise trial. The predetermined workload was to elicit 40%, 60%, or 80% of subjects' $VO_{2\ max,}$ calculated using from the initial visit using a regression analysis predicting oxygen utilization responses based on workload. The 20% differences in the intensities of each of the exercise trials were chosen to prevent any potential overlap in workloads within each subjects' exercise trials. The workload was set beforehand, but was occasionally adjusted after the protocol was initiated based on the subjects' metabolic responses. The reported workloads

were mean values calculated over the duration of the 30 minutes of exercise. The 20% differences in the intensities of each of the exercise trials were chosen to prevent any potential overlap in workloads within each subjects' exercise trials.

Heart rate was monitored every five minutes, and metabolic data was collected for three minutes at three points (minutes 7-10, 17-20, and 27-30) during the test to verify the workload met the prescribed intensity. After the 30 minutes of exercise at the prescribed workload was completed, the immediate post-exercise blood and saliva samples were collected (See Figure 4). After the blood sample was taken, subjects were allowed to actively cool down on the cycle ergometer, followed by a resting period. After 30 minutes recovery post-exercise, the last blood and saliva samples were collected. The subject was allowed to leave the laboratory after their heart rate reached approximately 100 bpm. This process was replicated for each of the exercise trials. For the resting control trial, the above procedures were repeated, but a 30-minute rest period was substituted for the exercise. These four trials were randomized and separated by a minimum of 72 hours.

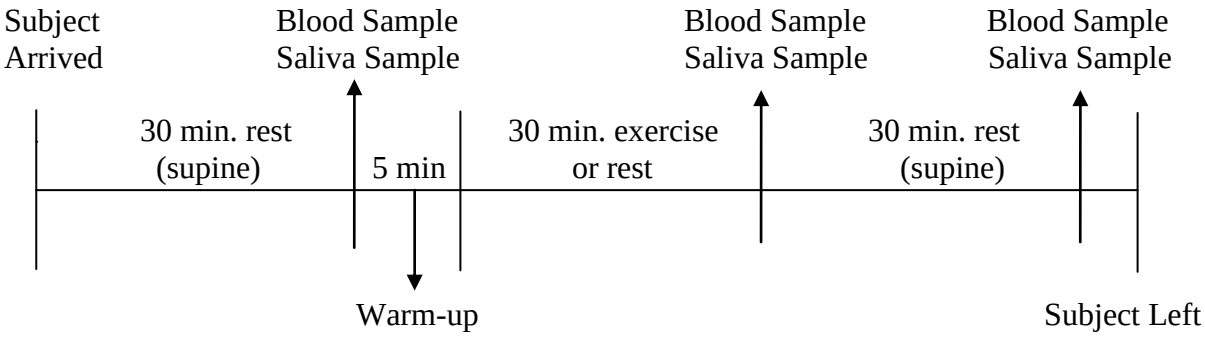

Figure 4. Overview of typical experimental trial.

Instrumentation

The height (cm) and body mass (kg) of each of the subjects were determined using a stadiometer (Perspectives Enterprises, Portage, MI) and a mechanical scale (Detecto, Webb City, MO). Body fat percentage was measured in triplicate at select sites (abdomen, chest, and thigh) using Cambridge Lange skinfold calipers (Cambridge Scientific, Cambridge, MA) and calculated using Jackson-Pollock method (Jackson et al., 1978). Respiratory gases were measured using a Parvo Medics TrueMax 2400 Metabolic System (Parvo Medics, Salt Lake City, UT, USA) and all exercise (maximal and submaximal) was completed on a Lode electronically braked ergometer (Lode, Groningen, The Netherlands). Heart rate was monitored during the exercise using a Polar HR monitor (Polar Model F1, Finland). Ratings of perceived exertion (RPE) were determined using Borg's 6-20 scale rate of perceived exercise scale (Borg, 1970).

Specimen procedures

Collection and storage

After placement of a catheter, blood samples (3 ml) were collected using a 3-cc syringe (Vanishpoint) and a 25 gauge needle (Retractable Technologies, Inc., TX, USA). All samples were immediately transferred into a sterile K^2 EDTA (purple top) tube (Vaccutainer) and were kept cool by being put on ice. For each exercise intensity, pre-trial, post-trial, and post-recovery hematocrit (Hct) were assessed in triplicate, using 75 mm microcapillary tubes (Fisher Scientific, PA, USA) sealed with Critoseal (Krakeler Scientific, Inc., Albany, NY). Samples were spun using the Adams MHCT II microhematocrit centrifuge (Becton

Dickinson, Franklin Lakes, NJ) for three minutes and subsequently read with a micro-hemoatocrit reader (International Equipment Company, Needham Heights, MA).

Resting (pre-trial), post-trial, and post-recovery hemoglobin (Hb) levels were also assessed in triplicate fashion from whole blood using the Stat-Site, WT-9" Hemoglobin Meter (Stanbio Laboratory, Boerne, TX). Using the mean Hct and Hb values, the changes in plasma volume were calculated for each trial using the Dill and Costill method (Dill & Costill, 1974). These changes indicated the effect of exercise induced fluid shifts on cortisol concentrations. Ultimately, the data analysis assessing serum cortisol concentrations were uncorrected for fluid shifts were used when comparing salivary and serum collection methods. After the initial whole blood analysis, the blood samples were spun at 3000 rpm and 4 degrees Celsius using a refrigerated centrifuge (IEC Cenra-8R, International Equipment Company, Needham Heights, MA) to separate plasma from erythrocytes. The plasma was pipetted into cryo-freeze tubes and stored at -80 degrees Celsius in an ultra-freezer (Revco Scientific, Inc., NC, USA).

Prior to collection of saliva samples, subjects were asked to rinse their mouths with water, spit, and then allow saliva to accumulate in the pool of their mouth. If saliva secretion needed to be stimulated, subjects were asked to chew on paraffin film. Accumulated saliva samples (minimum of 0.5 ml necessary) were collected from the subjects' mouths directly into a polypropylene cup. No more than 5 minutes past the desired time point was allowed to pass before saliva was collected. Collected samples were stored at -80 degrees Celsius.

Biochemical analysis

The stored serum and saliva samples were removed from storage allowed to warm to room temperature and then assessed for cortisol concentrations. Total serum cortisol concentrations were measured using a single-antibody, solid-phase radioimmunoassay (RIA) technique (Siemens Health Care, USA). Stored saliva samples were thawed and spun at 3000 rpm and 4 degrees Celsius. The saliva cortisol levels were measured using an expanded range high sensitivity enzyme immunoassay (EIA) kit (Salimetrics, State College, PA, USA).

It is important to note that free cortisol represents approximately 10% or less of the total cortisol amount found in the serum. This free portion of this hormone is what influences concentrations in the saliva and why absolute salivary concentrations are typically substantially lower than the total serum cortisol concentrations (Hellhammer et al., 2009).

Data analysis

Data analysis was performed using a computer based statistical software program (SPSS version 17.0, LEAD Technologies, Inc., Chicago, IL, USA). Mean and standard deviations for values for height, mass, age, body fat %, and $VO_{2\ max}$ were computed.

Separate, 4 x 3 (trial x sampling time), repeated measures analysis of variance (ANOVA) tests were used to determine if significant changes occurred in serum and saliva cortisol levels within each of the experimental trials (40%, 60%, 80% exercise intensity, control-rest). If the omnibus ANOVA analysis revealed significant findings, Tukey post-hoc tests were used to determine which means were significantly different. The significance level was set a priori at $\alpha \leq 0.05$.

Pearson product-moment correlations were used to assess the relationships between serum and salivary cortisol concentrations. The “r” values of the correlational analyses were used to compare pre-trial, post-trial, and post-recovery levels at each exercise intensity (40%, 60%, and 80%) and the control trial to determine how well the two methodologies compared at each respective time interval within subjects.

CHAPTER IV

RESULTS

Subject characteristics

Twelve moderate to high endurance-trained athletes participated in this investigation. Eleven of the twelve subjects completed all aspects of the study and one subject resigned from participation after completing two of the trials. The physical characteristics of the subjects (n = 12), expressed as mean ± standard deviation (SD) were as follows: age (years) = 22.0 ± 4.6; height (cm) = 174.9 ± 7.1; mass (kg) = 66.5 ± 9.5; BMI (kg/m^2) = 21.6 ± 1.9; and body fat (%) = 8.4 ± 2.1. All subjects were training for a minimum of three days per week for 60 minutes or more in the previous six months before they began the protocol. The subjects in the study had various backgrounds of training and sport participation, but primarily were experienced in endurance events, including cycling and running. To be included in the study, each of the subjects had to have a daily dietary intake consisting of at least 50% of calories (kcal) from carbohydrates (CHO). The subjects' mean CHO intake was 57.9% ± 3.7%. All of the subjects reported they had adhered to the experimental guidelines (see Methods) before the VO_{2max} session and experimental exercise and control trials (Trials I-IV).

VO_{2max}testing

Each of the subjects completed the VO_{2max} testing at least 7 days prior to the initiation of the experimental trials. All of the twelve subjects met the criteria for a valid test. The results of the VO_{2max} tests are displayed below in Table 1.

Measure	Value
Absolute $VO_{2\ max}$ (L/min)	3.84 ± 0.43
Relative $VO_{2\ max}$ (ml/kg/min)	58.2 ± 6.4
Peak Heart Rate (bpm)	194 ± 6
Peak RER	1.09 ± 0.04
Peak RPE	19 ± 1
Length of Test (min)	15.6 ± 2.0

Table 1. Mean values of the $VO_{2\ max}$ test for the subjects (n = 12). Values are expressed as mean ± standard deviation (SD).

Experimental testing trials

The results of the exercise trials indicate that the prescribed intensities were slightly higher than desired but did not overlap; therefore, the desired approximate 20% difference in intensities was achieved. Subjects also completed a trial during which no exercise was performed and served as a control trial for which to compare to the exercise trials. The results of the experimental testing trials are displayed below in Table 2.

Measure	**40% VO_2 max**	**60% VO_2 max**	**80% VO_2 max**
Workload (W)	95.0 ± 17.0	145.4 ± 18.8	181.9 ± 26.1
Heart Rate (bpm)	127 ± 14	156 ± 14	180 ± 8
VO_2 (ml/kg/min)	26.3 ± 3.3	37.8 ± 4.3	49.4 ± 4.6
% VO_2 max	45.3 ± 5.0	65.2 ± 7.7	85.0 ± 5.8
RPE	10 ± 2	13 ± 2	16 ± 1

Table 2. Results (average over 30 min. of exercise) of select variables measured during each respective exercise trial (n = 12). Values are expressed as mean ± standard deviation (SD).

Plasma volume changes

Plasma volume (PV) changes as a result of the experimental trials were calculated from mean Hb and Hct values collected pre-trial, post-trial, and post-recovery. The greatest mean PV shifts from pre-trial to post-trial as well as from pre-trial to post-recovery occurred during the 80% experimental trial. Table 3 displays these changes.

Trial	**Hct (%)**			**Hb (g/dL)**			**Δ Pre-Trial to Post-Trial**	**Δ Pre-Trial to Post-Recovery**
	Pre	**Post**	**Rec**	**Pre**	**Post**	**Rec**		
Control	38.9 ± 0.6	39.8 ± 0.4	39.3 ± 0.4	14.1 ± 1.2	14.8 ± 1.0	14.6 ± 1.0	-6.1 ± 3.3	-4.2 ± 2.0
40%	37.7 ± 0.3	40.4 ± 0.4	39.3 ± 0.4	14.2 ± 0.9	15.3 ± 0.9	14.5 ± 0.9	-11.1 ± 4.2	-5.1 ± 4.4
60%	38.2 ± 0.3	41.0 ± 0.3	39.8 ± 0.4	14.0 ± 1.0	14.8 ± 0.7	14.5 ± 0.8	-10.4 ± 4.4	-6.1 ± 5.0
80%	38.1 ± 0.4	41.2 ± 0.4	39.9 ± 0.4	14.1 ± 0.9	15.5 ± 1.5	14.8 ± 0.9	-14.2 ± 5.6	-8.0 ± 4.0

Table 3. Mean Hct, Hb values for pre-trial, post-trial and post-recovery with corresponding plasma volume changes (% changes) from pre-trial to post-trial and from pre-trial to post-recovery (n = 12). All values are expressed as mean ± standard deviation (SD).

Cortisol analysis

The mean (± SD) serum and salivary cortisol responses to the different experimental trials analyzed at the three respective sampling time points are highlighted in Tables 4 and 5. These hormonal values were not corrected for PV changes.

Serum cortisol

Trial	***Serum Cortisol Concentrations (µg/dL)***		
	Pre-Trial	**Post-Trial**	**Post-Recovery**
Control	12.2 ± 5.4	9.8 ± 4.3	10.5 ± 4.9
40% $VO_{2\ max}$	14.3 ± 7.0	13.8 ± 6.0	10.9 ± 4.3
60% $VO_{2\ max}$	14.0 ± 4.5	16.2 ± 5.1	11.1 ± 5.1**
80% $VO_{2\ max}$	12.2 ± 4.4	20.5 ± 6.7*	20.5 ± 7.8*

Table 4. Mean serum cortisol concentrations for each respective experimental trial (n=12). Values are expressed as mean ± standard deviation (SD).
*Significant difference from respective pre-trial ($p < 0.05$).
**Significant difference from respective post-trial ($p < 0.05$).

Control/resting trial: For the control/resting trial, serum cortisol concentrations decreased slightly during the first 30 minutes of rest and then increased slightly during the last 30 minutes of rest. However, these changes were not significant.

40% $VO_{2\ max}$ exercise trial: The cortisol concentrations decreased from pre-trial to post-trial and also decreased from post-trial to post-recovery. However, these changes were not significant.

60% $VO_{2\ max}$ exercise trial: Cortisol concentrations increased slightly from pre-trial to post-trial, but these changes were not significant. However, from post-trial to post-recovery,

cortisol responses decreased by 31.2% ($p = 0.006$). The peak cortisol response in this trial occurred at the post-trial sampling time.

80% $VO_{2\ max}$ exercise trial: Cortisol responses increased significantly from pre-trial to post-trial ($p \leq 0.001$) as well from pre-trial to post-recovery ($p \leq 0.001$). These significant increases represented identical 40.4% elevations from pre-trial concentrations at each of the respective time intervals (post-trial and post-recovery). The peak cortisol response in this trial was at the post-trial sampling time (i.e. when concentrations were expressed to the second decimal place); although there was no difference between mean values at post-trial and post-recovery sampling times.

Salivary cortisol

Trial	***Salivary Cortisol Concentrations (µg/dL)***		
	Pre-Trial	**Post-Trial**	**Post-Recovery**
Control	0.256 ± 0.168	0.251 ± 0.148	0.224 ± 0.200
40% $VO_{2\ max}$	0.268 ± 0.204	0.213 ± 0.157	0.190 ± 0.138
60% $VO_{2\ max}$	0.189 ± 0.114	0.293 ± 0.133	0.259 ± 0.165
80% $VO_{2\ max}$	0.170 ± 0.073	0.349 ± 0.216*	0.460 ± 0.251*

Table 5. Mean (± SD) cortisol concentrations for each respective experimental trial. Values are expressed as mean ± standard deviation (SD).
*Significant difference from respective pre-trial ($p < 0.05$).

Control/resting trial: For the control/resting trial, salivary cortisol concentrations decreased slightly during the first 30 minutes of rest and again from minute 60 to minute 90 of rest. However, none of these changes were significant.

40% $VO_{2\ max}$ exercise trial: The cortisol concentrations in the saliva in this trial were very similar to the responses during the control trial. Levels decreased slightly immediately post-trial and again dropped slightly from post-trial to post-recovery. Once again, none of these changes in salivary cortisol levels were significant.

60% $VO_{2\ max}$ exercise trial: Cortisol concentrations in the saliva in this trial mirrored the responses of those in the serum during the same experimental trial, but were not as robust. Responses increased slightly from pre-trial to post-trial and again from post-trial to post-recovery. However, none of these salivary changes were significant. The peak cortisol response in this trial occurred at the post-trial sampling time.

80% $VO_{2\ max}$ exercise trial: During the 80% trial, salivary cortisol responses increased significantly from pre-trial to post-trial ($p = 0.01$) and also from pre-trial to post-recovery ($p \leq 0.001$). No other significant changes were noted. The peak cortisol response in this trial occurred at the post-recovery sampling time.

Cumulative serum versus salivary cortisol responses

To determine how well serum-saliva concentrations tracked, a composite correlation analysis (Pearson Product Moment) was used to assess the aggregate relationship between the two sampling methodologies. Figure 5 displays this relationship, where the correlation coefficient for the two methods was $r = 0.548$, which was significant ($p < 0.005$).

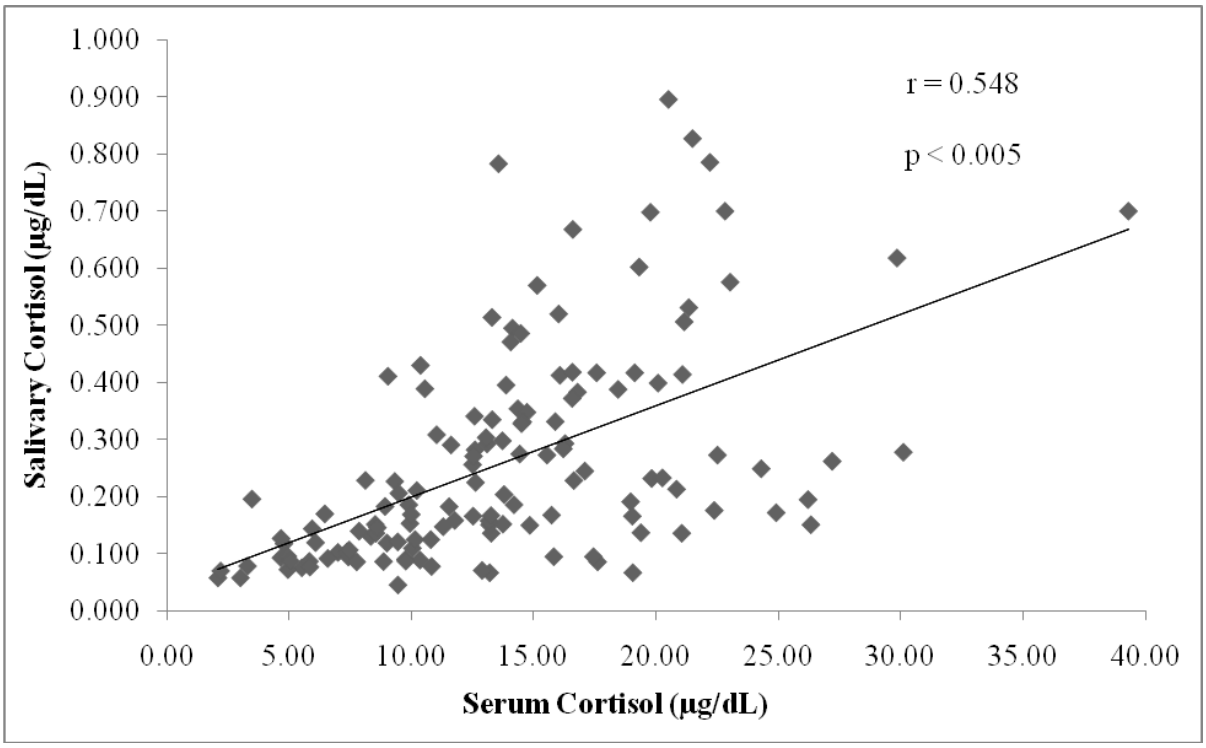

Figure 5. The relationship between matched serum and salivary cortisol responses across all experimental conditions and time intervals.

Serum versus salivary cortisol responses within sessions

Pearson Product Moment correlations were used to compare sampling methods within each experimental trial (40%, 60%, 80% $VO_{2\ max}$ and control) at each specific time point during a trial. These correlations measured the associations between the matched pairs for serum and saliva within the respective trial and sampling time point. These results are highlighted in Table 6. The highest correlations between the methods were in the control and 40% VO_{2max} trials when comparing across experimental trials. When evaluating the two methods across the sampling times, the highest correlations tended to be during the post-recovery time point.

Trial	*Pearson Product Moment Correlation Coefficients*		
	Pre-Trial	**Post-Trial**	**Post-Recovery**
Control	r = 0.627*	r = 0.797*	r = 0.476
40% $VO_{2\ max}$	r = 0.826*	r = 0.591*	r = 0.706*
60% $VO_{2\ max}$	r = 0.103	r = 0.351	r = 0.644*
80% $VO_{2\ max}$	r = 0.047	r = 0.053	r = 0.426

Table 6. Pearson Product Moment correlation coefficients for serum vs. salivary cortisol concentrations for each respective experimental trial.
*Indicates significant correlations ($p < 0.05$)

CHAPTER V

DISCUSSION

Introduction

The primary purpose of this study was to investigate the associations between serum and salivary cortisol responses at three different exercise intensities (40%, 60%, and 80% of $VO_{2\ max}$), to determine if salivary concentrations could be an accurate method to assess the body's response to exercise. The hypothesized outcome was that there would be significant correlations between the serum and salivary cortisol levels at all sampling times. It was also expected that the peak salivary cortisol response when compared to the peak serum response within the 60% and 80% exercise trials would occur at a later sampling time. This was predicted since the movement of free cortisol from the capillaries into the saliva is passive; thus, the peak response of the hormone due to the exercise was expected to be delayed compared to the peak responses in serum concentrations.

The discussion in this chapter is organized into several sections. First, there is a discussion about the subjects' physiological responses to the exercise trials, focusing on the how close the subjects were to the desired exercise intensities. Second, both the subjects' serum and salivary cortisol responses are discussed, analyzing how they compared to other related exercise studies. Third, there is a discussion of how the associations between the methods compared to previous studies which evaluated the relationship between these methods. Fourth, the peak responses of cortisol in the moderate and high intensity trials are

compared and addressed. Finally, limitations and conclusions of the present study are discussed.

Exercise responses

As highlighted in Table 2, the exercise trials produced the desired physiological responses from the subjects. A regression equation was used to predict the workload that would bring about the prescribed intensities and the workload was adjusted at 10 minutes and/or 20 minutes into the trial if the VO_2 responses were higher or lower than what was stipulated. Ultimately, the average intensities over the 30 minutes of exercise were slightly higher than what was predicted, 45.3 ± 5.0%, 65.2 ± 7.7%, and 85.0 ± 5.8%, respectively. However, none of the responses overlapped. This was also apparent when looking at the heart rates (95 ± 17 bpm, 145 ± 19 bpm, and 182 ± 26 bpm, respectively), and RPE values reported by the subjects during the trials (10 ± 2, 13 ± 2, and 16 ± 1, respectively). These findings suggest the desired effect for the experimental exercise protocol was achieved. Hematocrit and hemoglobin values were measured within each blood sample in order to calculate plasma volume shifts (Dill & Costill, 1974). These hematological measures were assessed to determine if the subjects were well hydrated at the start of each trial for each of the experimental trials and normal hemodynamic responses to exercise occurred. Results support that all subjects were adequately hydrated and their fluid responses to exercise were normal and in agreement with the literature (Hagen et al. 1980).

Cortisol responses

Serum

All serum values were within the normal expected range of values for the all the respective measurement times and experimental trials (Siemens Health Care, USA). During the control and the 40% exercise trials, serum cortisol responses decreased from pre-trial levels both immediately after rest or exercise as well as at the post-recovery from rest or exercise. The decrease in cortisol during the rest of the control trial is reflective of the normal circadian pattern for cortisol (Kerrigan et al., 1993). The 40% exercise trial results are consistent with previous studies analyzing the threshold-intensity effect. That is, while there are some divergent findings, most literature has supports a ~60% exercise intensity threshold is necessary to elicit a significant increase in blood cortisol (Davies & Few, 1973; Hill et al., 2008).

During the 60% exercise trial, cortisol responses did not significantly increase as was expected (Davies & Few, 1973). While levels were elevated by 16% compared to pre-trial levels, the increase was not significant at this intensity. This may be explained by the high aerobic fitness level (>50 ml/kg/min) of the subjects. Persons who are highly trained tend to have a higher intensity threshold to provoke an increase in cortisol (Bloom et al., 1973; Viru & Viru, 2004).

Another unexpected finding during the 60% trial was that the cortisol responses significantly decreased from post-trial to post-recovery. Levels were expected to decrease in the serum during recovery, but it was projected that the levels would not significantly drop to below those measured before the exercise. Why this change occurred is unclear, but it could

reflect the circadian pattern of cortisol secretion and/or a natural decline in the hormonal levels as a function of feedback regulation (Few et al., 1970; Kerrigan et al., 1993).

During the 80% trial, cortisol responses significantly increased from rest (pre-trial) and remained elevated during the 30 minute recovery from the exercise trial. This finding concurs with several studies which used similar designs to the present study (Bloom et al., 1973; Davies & Few, 1973; Hill et al., 2008). This prolonged elevation in cortisol during the recovery period has been previously found following intensive, stressful exercise and demonstrates hormonal changes can last well past the cessation of exercise (Daly et al., 2004).

Saliva

All saliva values were within the normal expected range of values for the all the respective measurement times and experimental trials (Salimetrics, USA). For the control session and the 40% exercise trial, salivary cortisol responses followed the same trend as serum cortisol; that is, levels decreased from pre-trial to post-trial and remained depressed after the 30 minute recovery trial. However, these changes were not significant. For the 60% trial, levels did not significantly increase from that of the pre-trial measurement. Despite an average increase of 35% in the 60% trial, the increase was not significant due to the high variability of responses within the subjects. Levels still remained elevated (23%) at post-recovery compared to pre-trial unlike the significant decrease evident in the serum concentrations within this trial.

The intensity-threshold effect was clear in the 80% exercise trial as levels significantly increased from pre-trial to post-trial. This is in agreement with previous studies

using saliva sampling assessing cortisol responses to varying exercise intensities. For example, Jacks and colleagues demonstrated the threshold-intensity effect in salivary cortisol utilizing 3 different exercise intensities (mean intensities of 44.5 %, 62.3, and 76.0 %). During the highest-intensity exercise session, cortisol was significantly higher after the exercise session ($p = 0.004$) compared to baseline (Jacks et al., 2002). Kivlighan and colleagues also used saliva as the sampling specimen for obtaining cortisol responses to the anticipation of a competitive (high intensity) exercise session as well as during the recovery period after the exercise. Levels significantly increased from baseline in all groups (competitive and non-competitive males and females) after the exercise session (Kivlighan et al., 2005).

Some studies employing resistance exercise have also demonstrated significant increases in cortisol responses measured in the saliva. For example, McGuigan and colleagues found that high intensity (75%) resistance exercise resulted in significant ($p < 0.05$) increases in salivary cortisol compared to resting levels. Low intensity resistance exercise (30%) did not result in any significant increases (McGuigan et al., 2003). Also, Paccotti and colleagues found that there was a significant increase ($p < 0.05$) in salivary cortisol levels in competitive athletes immediately after an acute bout of high intensity resistance training. Cortisol levels also remained significantly elevated at 90 and 120 minutes after termination of the exercise (Paccotti et al., 2005).

The unique response in the present study was salivary concentrations were highest (and possibility still increasing) after 30 minutes of recovery from the 80% trial. This was evidenced by 31% higher average concentrations comparing post-trial to post-recovery. This finding is divergent from that of Jacks et al. who also used recovery sampling as part of their

experimental design in measuring cortisol in saliva and found cortisol levels remained elevated from rest after 20 minutes of recovery from a 60 minute exercise bout on a cycle ergometer, but were still slightly lower than levels measured immediately post-exercise (Jacks et al., 2002). The relatively long exercise duration of 60 minutes of this latter study may have allowed sufficient time for the diffusion of the free cortisol levels into the saliva during the recovery. Thus, there was not as great a potential for a temporal lag between responses. The present study utilized a shorter exercise duration which may have been an inadequate amount of time for saliva levels to completely reflect the blood levels (Ben-Aryeh et al., 1989).

Comparison of serum to salivary cortisol

The composite correlation coefficient between serum and saliva cortisol was 0.548, which was significant ($p < 0.005$). This relationship is displayed in Figure 7. The high level of significance can be partially attributed to the large sample size ($n = 135$). While this is a moderate correlation and it appears the two methods track well, this correlation coefficient was lower than what was expected based on previous literature. Kirschbaum and colleagues' reported correlation coefficients between cortisol in saliva and cortisol in serum ranging from $r = 0.71$ to $r = 0.96$ (Kirschbaum et al., 1994). Umeda and Iwaoka's investigation also reported a strong correlation coefficient of $r = 0.893$ ($n = 10$; $p < 0.001$) between serum and saliva, which was significant considering the small sample size Umeda & Iwaoka, 1981). Goodyer and colleagues also found correlations that were significant ($r = 0.81$; $p < 0.005$) but had a large sample size ($n = 284$) which may have contributed to the relationship (Goodyer et al., 2001). However, Gonzansky and colleagues yielded results with less robust correlations,

with a coefficient between serum and saliva cortisol of r = 0.60, which was significant (Gonzansky et al., 2005).

The primary difference between the present study and these previous studies are the means of stimulation of the HPA axis. Many of the above studies measured cortisol levels in a resting state and not using exercise as a stressor. For example, in Umeda and Iwaoka's (1981) investigation, instead of using exercise as a stimulus to the HPA axis, they used ACTH stimulation to elevate levels of cortisol (Umeda & Iwaoka, 1981). Since there is more variance using exercise as a stressor to the HPA axis compared to synthetic augmentation (Paccotti et al., 2007), this may partially explain the difference in the strength of the associations between this study and the present study.

Comparison of serum and salivary cortisol during exercise

A major aim of this study was to evaluate the relationship between serum and salivary cortisol responses to 30 minutes of exercise of different intensities at different sampling times. To assess this outcome, correlations for each of the respective trials (control, 40%, 60%, 80%) and sampling times (pre-trial, post-trial, and post-recovery) were used to gauge how well the methods were associated with one another. Table 6 lists each of the correlations and indicates which correlations are significant within a particular experimental trial.

Pre-trial concentrations were highly correlated before the control trial as well as the 40% trial. However, correlations were very weak at the same time point for the 60% and 80% trial, which was unexpected. All pre-trial resting cortisol levels were expected to be correlated based upon the studies discussed in the section above. This finding of low

correlations for the 60% and 80% trials at rest is difficult to explain. Methodological flaws in the assay procedures are not a likely factor. All assay coefficients of variation (CV) were at less than 10% for serum and saliva determinations. Also, all assay quality control standards were within acceptable ranges of variance (5-10%). At this time, no explanation for the lack of significant correlations at rest in the 60% and 80% trial can be given.

Post-trial hormone levels were significantly correlated within the control, 40% and 60% trials but not in the 80% trial. The lack of association between the serum-saliva specimens after highest intensity exercise trial was anticipated, and may be due to the proposed diffusion rate differences between the blood-saliva at higher hormonal concentrations (Ben-Aryeh et al., 1989; Vining et al., 1983). The significant correlations immediately after exercise agree with some previous studies. For example, Thomasson and colleagues compared plasma and saliva hormones in response to exercise, but only measured cortisol response to an exercise intensity of 50-55% $VO_{2\,max}$. There were significant correlations ($p < 0.05$) at rest; but the correlations measured throughout the steady-state exercise bout were low to moderate ($r = 0.35$), although still significant ($p < 0.02$) (Thomasson et al., 2009). Also, Cadore and colleagues found that correlation between serum and salivary cortisol responses after resistance exercise were somewhat stronger ($r = 0.62$, $p = 0.001$) than reported here. However, this resistance exercise was of an undefined intensity; therefore, it is difficult to make valid comparisons to the present study (Cadore et al., 2008).

In the present study, the post-recovery levels tended to have higher correlations across all of the experimental trials when compared to the other sampling time points. However, only the 40% ($r = 0.706$) and 60% ($r = 0.644$) trials were at a significant level, whereas after the 30 minute recovery, the control and 80% correlations only approached

significance (p = 0.10-0.15). Few studies have examined the relationship between serum-saliva cortisol concentrations during the recovery from exercise. Neary and colleagues compared serum and saliva concentrations one day into the recovery from an exercise training session and found that serum and saliva concentrations were highly associated, r = 0.99 (Neary et al., 2002). However, the long recovery period (1 day) was significantly more time than the present study (30 minutes), which make comparisons between the two studies somewhat problematic. The lack of significance between the serum-saliva measurements after the 30 minute recovery from the 80% trial is likely due to the fact that salivary concentrations were still increasing compared to the leveling off seen in the serum. The lack of a significant correlation in the recovery from the 80% trial may be evidence for the hypothesized diffusion rate differences between salivary cortisol responses compared to serum responses.

Peak responses

When comparing peak responses in serum and saliva in the 60% trial, both occurred immediately after the exercise, meaning that the mean responses mirrored each other even though the concentrations were not significantly correlated at this sampling time. This was different than what was hypothesized because it was predicted that concentrations in the saliva would peak at a later time point (post-recovery) in this trial compared to serum. This may be explained by the fact that since cortisol responses were not significantly augmented with either sampling technique during the 60% trial, the magnitude of increase was not large enough to elicit a delay in the response of the saliva compared to serum.

In the 80% trial, the peak response occurred at a later time point (post-recovery) when saliva was compared to serum (post-trial). This is in agreement with the second research

hypothesis and supports that salivary cortisol responses may be delayed due to the passive movement of the hormone from the blood into the saliva (Ben-Aryeh et al., 1989). This delayed response may be due to the lack of blood flow during exercise to the mouth area (i.e. salivary glands) where saliva is produced. Blood is shunted to the working skeletal muscle during exercise. The reduced blood flow at the mouth could decrease the cortisol available to cross from the capillaries to the saliva through the passive diffusion process. However, once the exercise is complete, the blood is steadily redistributed from the muscle to the rest of the body (Brooks et al., 2000). During recovery from exercise the amount of blood flow to the mouth would slowly return to normal, thereby increasing the amount of free cortisol available to diffuse from the blood into the saliva. This delayed response accentuates the need to include sampling during the recovery from intense exercise when assessing salivary cortisol responses since peak salivary levels occur at a later time point than in serum for the 80% high intensity exercise.

Limitations of study

There are potential limitations and confounding factors in this investigation which may have impacted the results and potentially limit the reliability and validity of the findings. First, both small sample size (n = 12) and the demographics of the subjects (trained male athletes 18-30 years of age) make it difficult to generalize the findings to other populations. Second, some subjects completed their trials in the morning while others completed their trials in the afternoon, potentially causing differing responses between subjects; although, each respective subject did consistently replicate the time of day for each of their trials. Third, the researchers relied on the truthfulness of the subjects in providing background

information and adhering to experimental compliance procedures, including: medical information, training history, dietary records, acute training (no strenuous exercise in the 24 hours prior to the trials), diet (4 hours post prandial, and no alcohol, NSAIDs, or caffeine in the previous 8 hours) and stress states (forthright answers in the REST-Q questionnaire). Inaccuracies in the information and/or procedures may have introduced systematic error into the study and confounded outcomes.

The collection of samples of serum and saliva may have also introduced error into study. The drawback of blood sampling is that venipuncture can elicit stressful responses that could lead to rapidly elevated blood cortisol levels. This 'white-coat' effect cannot be disregarded if testing is carried out where both the testing situation and personnel are unfamiliar and in situations where subjects remain in anticipation of the venipuncture (Levine et al. 2007). Although in the present study, subjects rested for 30 minutes before the first blood sample was taken as well as experienced the procedure on four separate occasions, which might have mitigated some of this effect. Finally, the sampling procedure for saliva can be problematic. Saliva which is provided after eating or drinking substances with low pH interferes with assay results; although, the current subjects were supposed to have been 4 hours post-prandial as noted above. Also, the presence of blood in saliva due to oral lesions can affect salivary assay results (Schwartz & Granger, 2004). The current subjects did not have their mouths inspected prior to saliva collections to determine if this could have been a problem. The researcher tried to control all of these factors through sufficient planning, dialogue with the subjects, and collection in controlled environment; nonetheless, errors and oversights may still have occurred.

Summary

The present study is one of the only studies comparing serum and saliva cortisol levels at varying exercise intensities and recovery from exercise. Collectively, the results suggest salivary measurements of cortisol can, in some circumstances mirror those in serum in response to exercise. However, the present data do not support that salivary cortisol and serum cortisol perfectly track one another at different intensities of exercise.

As the saliva cortisol measurement method becomes more established, it may prove to be very beneficial to the field of sports physiology. Assessing cortisol allows for the opportunity to collect the samples without medical personnel, it minimizes stress in the collection process, permits frequent and rapid sampling, and facilitates sampling in a variety of environments. These advantages are specifically relevant for athletes, since practice and competitions take place outside of the laboratory environment.

CHAPTER VI

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Despite the limitations of this study, there are many insights that can be gained from the findings. When analyzing specific intensities and time points, correlations between the serum-saliva sampling techniques were significant after recovery from exercise at 40% and 60% of $VO_{2\ max}$. The intensity threshold effect was evident in both sampling methods during the 80% intensity, suggesting that salivary concentrations are similar to serum comparing across subjects. Finally, there was evidence for a hypothesized delay in the peak levels measured in the saliva after recovery from the high intensity (80%) trial. However, there needs to be more research completed on this topic, utilizing more subjects and perhaps introducing a longer period of recovery into the experimental protocol, especially during high intensity exercise. Nonetheless, the findings of this study suggest that salivary measurement of cortisol may be a viable option for assessing this hormone as long as research personnel are aware of sources of variance and differences from serum sampling.

Salivary cortisol sampling may be preferable to serum. Since cortisol is a stress hormone, measuring this hormone from blood sampling introduces stress in the sampling process itself, which is potentially a major confounding factor (Levine et al., 2007). Collection of saliva, on the other hand, is less invasive, is much easier than setting up the blood draws and requiring a phlebotomist to be present. However, particularly when using this sampling technique in exercise studies, being aware of confounding factors and sources

of variance is crucial when drawing conclusions. Monitoring levels of this hormone throughout and between training cycles may lend insight into the stress that an exercise program provides and may enlighten coaches, athletes, and research personnel to the optimal stimuli to promote adaptation to training.

Conclusions

Research hypothesis #1: *There will be significant correlations between serum and salivary cortisol concentrations at the pre-trial, post-trial, and post-recovery sampling time within each of the experimental trials (control, 40%, 60% and 80% $VO_{2\ max}$ exercise).* This hypothesis was rejected since only 6 of the 12 correlations were significant.

Research hypothesis #2*:* *The peak saliva cortisol response when compared to the peak serum response within the 60% and 80% exercise trials will occur at a later sampling time.* This hypothesis was rejected for the 60% exercise, but accepted for the 80% exercise. Both the serum and saliva peak responses occurred immediately post-trial in the 60% exercise trial, which did not support the research hypothesis. However, in the 80% exercise, trial, the peak response for salivary cortisol occurred during a later sampling time (30 minutes; post-recovery) than with serum (post-trial), which agreed with the research hypothesis.

APPENDICES

A. Medical and training history

B. Physical screening

C. Informed consent form

D. Data collection sheets

E. Assay information

APPENDIX A

MEDICAL HISTORY

Department of Exercise and Sport Science
Medical History

Subject: _____ ID: _____ Telephone:_____

Address:_____

Occupation:_____ Age:_____

YES NO

Patient History

1. How would you describe your general health at present?

Excellent_____ Good_____ Fair_____ Poor_____

2. Do you have any health problems at the present time? _____ _____
3. If yes, please describe:_____
_____
4. Have you ever been told you have heart trouble? _____ _____
5. If yes, please describe:_____
_____
6. Do you ever get pain in your chest? _____ _____
7. Do you ever feel light-headed or have you ever fainted? _____ _____
8. If yes, please describe:_____
_____
9. Have you ever been told that your blood pressure has been elevated? _____ _____
10. If yes, please describe:_____
_____
11. Have you ever had difficulty breathing either at rest or with exertion? _____ _____
12. If yes, please describe:_____
_____
13. Are you now, or have you been in the past 5 years, under a doctor's care for any reason?
_____ _____
14. If yes for what reason?_____
_____
15. Have you been in the hospital in the past 5 years? _____ _____
16. If yes, for what reason?_____
_____
17. Have you ever experienced an epileptic seizure or been informed that you have epilepsy?
_____ _____
18. Have you ever been treated for infectious mononucleosis, hepatitis, pneumonia, or another infectious disease during the past year? _____ _____
19. If yes, name the disease:_____

20. Have you ever been treated for or told you might have diabetes? _____ _____
21. Have you ever been treated for or told you might or low blood sugar? _____ _____
22. Do you have any known allergies to drugs? _____ _____
23. If so, what?_____

_____

24. Have you ever been "knocked-out" or experienced a concussion? _____ _____
25. If yes, have you been "knocked-out" more than once? _____ _____
26. Have you ever experienced heat stroke or heat exhaustion? _____ _____
27. If yes, when?_____

_____

28. Have you ever had any additional illnesses or operations? (Other than childhood diseases) _____ _____
29. If yes, please indicate specific illness or operations:_____

_____

30. Are you now taking any pills or medications? _____ _____
31. If yes, please list:_____

_____

32. Have you had any recent (within 1 year) difficulties with your:
    a. Feet _____ _____
    b. Legs _____ _____
    c. Back _____ _____

Family History

33. Has anyone in your family (grandparent, father, mother, and/or sibling) experienced any of the following?
    a. Sudden death _____ _____
    b. Cardiac disease _____ _____
    c. Marfan's syndrome _____ _____

Mental History

34. Have you ever experienced depression? _____ _____
35. If yes, did you seek the advice of a doctor? _____ _____
36. Have you ever been told you have or has a doctor diagnosed you with panic disorder, obsessive-compulsive disorder, clinical depression, bipolar disorder, or any other psychological disease? _____ _____
37. If yes, please list condition and if you are currently taking any medication.

Condition Medication

_____

_____

_____

Bone and Joint History

34. Have you ever been treated for Osgood-Schlatter's disease? _____ _____
35. Have you ever had any injury to your neck involving nerves or vertebrae? _____ _____

36. Have you ever had a shoulder dislocation, separation, or other injury of the shoulder that incapacitated you for a week or longer? _____ _____
37. Have you ever been advised to or have you had surgery to correct a shoulder condition? _____ _____
38. Have you ever experienced any injury to your arms, elbows, or wrists?_____ _____
39. If yes, indicate location and type of injury:_____
_____
40. Do you experience pain in your back? _____ _____
41. Have you ever had an injury to your back? _____ _____
42. If yes, did you seek the advice of a doctor? _____ _____
43. Have you ever been told that you injured the ligaments or cartilage of either knee joint? _____ _____
44. Do you think you have a trick knee? _____ _____
45. Do you have a pin, screw, or plate somewhere in your body as the result of bone or joint surgery that presently limits your physical capacity? _____ ____
46. If yes, indicate where:_____
_____
47. Have you ever had a bone graft or spinal fusion? _____ _____

Activity History

48. During your early childhood (to age 12) would you say you were:
    Very active ____ Quite active____ Moderately active____ Seldom active____
49. During your adolescent years (age 13-18) would you say you were:
    Very active ____ Quite active____ Moderately active____ Seldom active____
50. Did you participate in:
    a. Intramural school sports? _____ _____
    b. Community sponsored sports? _____ _____
    c. Varsity school sports? _____ _____
    d. Active family recreation? _____ _____
51. Since leaving high school, how active have you been?
    Very active ____ Quite active____ Active____ Inactive____
52. Do you participate in any vigorous activity at present? _____ _____
53. If yes, please list:

Activity	Frequency	Duration	Intensity

54. How would you describe your present state of fitness?
Excellent_____ Good_____ Fair_____ Poor_____
55. Please list the type(s) of work you have been doing for the previous ten years:

Year	Work	Indoor/Outdoor	Location (city/state)

56. Whom shall we notify in case of emergency?
    Name:_____
    Phone: (Home)_____ (Work)_____
    Address:_____
57. Name and address of personal physician:_____
_____
_____

All of the above questions have been answered completely and truthfully to the best of my knowledge.

Signature:_____ Date:_____

APPENDIX B

PHYSICAL SCREENING

Department of Exercise and Sport Science
Physical Examination Screening

Name: _____ Age: _____ Gender: _____

Please respond to each of the following in writing.

Pulse rate and regularity: _____ ECG Interpretation: _____

Blood Pressure:
Supine: _____ Sitting: _____ Standing (Left side): _____
Squat: _____ Standing (Right side): _____

Marfan Syndrome evaluation: (Δ BP, Physical Char.) _____

Palpation of Pulses: Carotid: _____ Radial: _____ Pedal: _____

Auscultation of the Lungs:
Back: Lower: _____ Middle: _____ Upper: _____
Front: Middle: _____ Upper: _____

Auscultation of Heart Sounds (Supine, Standing, Squatting)
Non-Specific HS: _____/_____/_____
Murmur: ____ Gallop: ____ Click: ____ Rub: ____ Click w/ Murmur: ____

Bruits: Carotid: _____ Abdominal: _____

Edema: Abdominal: _____ Calf: _____ Pedal: _____

Tenderness: Abdominal: _____ Other: _____

Xanthoma or xanthelasm: _____

Medical/Family History:
High Blood Pressure: _____ Diabetes: _____ CHD/CAD: _____

Last examination w/ physician: _____
Medications (prescription/ counter): _____

Examiner: _____ Date: _____

APPENDIX C

Informed Consent

University of North Carolina-Chapel Hill
Consent to Participate in a Research Study
Adult Participants
Social Behavioral Form

IRB Study #__09-1704_____
Consent Form Version Date: 9/28/2009

Title of Study: The Relationship between Plasma and Salivary Cortisol Levels in Response to Different Exercise Intensities

Principal Investigator: Mitch D. VanBruggen
UNC-Chapel Hill Department: Exercise and Sport Science (EXSS)
UNC-Chapel Hill Phone number: 763-670-7878
Email Address: vanbrugg@email.unc.edu

Faculty Advisor: Dr. Anthony C. Hackney
Study Contact telephone number: 919-962-0334
Study Contact email: ach@email.unc.edu

What are some general things you should know about research studies?

You are being asked to take part in a research study. To join the study is voluntary. You may refuse to join, or you may withdraw your consent to be in the study, for any reason, without penalty.

Research studies are designed to obtain new knowledge. This new information may help people in the future. You may not receive any direct benefit from being in the research study. There also may be risks to being in research studies.

Details about this study are discussed below. It is important that you understand this information so that you can make an informed choice about being in this research study. You will be given a copy of this consent form. You should ask the researchers named above, or staff members who may assist them, any questions you have about this study at any time.

What is the purpose of this study?

The purpose of this research study is to learn about the relationship between blood and saliva cortisol levels at rest and in response to different exercise intensities to determine if salivary concentrations can be an accurate method to assess the body's stress response to exercise.

You are being asked to be in the study because you are an endurance-trained male athlete.

Are there any reasons you should not be in this study?

You should not be in this study if you do not exercise regularly; consume a diet chronically low in carbohydrates; have a prior history of hormonal disorders; have a mental illness; or engage in chronic non-steroidal anti-inflammatory (i.e. ibuprofen) drug use.

How many people will take part in this study?

If you decide to be in this study, you will be one of approximately twelve people in this research study.

How long will your part in this study last?

You will be asked to come to the Applied Physiology Laboratory (APL) in Fetzer Gymnasium for approximately 90-120 minutes on 5 separate occasions. These testing sessions will be separated by a minimum of 72 hours. More specific scheduling will be done after you agree to participate in the study, although each session will occur at the same time of day (within 30 minutes). After your final exercise session, your participation in the study will be completed.

What will happen if you take part in the study?

You will be asked to report to the Applied Physiology Laboratory (APL) in Fetzer Gymnasium at the University of North Carolina-Chapel Hill on five separate occasions. You should maintain a consistent food intake throughout the duration of your participation. Also, you are asked to come to the laboratory having not exercised for 24 hours, not eaten for the previous 4 hours, and having consumed no caffeine or alcohol for 8 hours prior to the session. You must wear athletic clothing and shoes, or appropriate cycling gear to each exercise session. A locker room will be provided should you need to change clothes.

First visit to the laboratory:

1. During the first visit, you will learn specific details about the study, read and sign the informed consent form, and have a brief discussion on dietary intake and training history. You will also complete a questionnaire to measure your stress levels. You will then undergo a familiarization process with the bike apparatus, blood and saliva collection procedures, and have your height, and body weight recorded. You will also have your body fat percentage measured using skinfold calipers.
2. Next, your blood pressure will be taken, and then you will have 10 electrodes placed on your chest in order to acquire a 12-lead resting electrocardiogram to determine if cardiovascular problems exist.
3. If the ECG recording is normal, you will be properly fit with a mouthpiece (used to measure your respiratory gases), and a heart rate monitor. A resting oxygen uptake will be assessed to make sure that the values are normal and the metabolic system is functioning properly. If everything is normal, you will be instructed to warm up on the bike for 5 minutes at a very light workload. This light warm-up will be followed by 5 minutes of stretching.
4. After the warm-up, you will perform a bicycling graded exercise test, to determine your maximal aerobic capacity ($VO_{2\ max}$). During the test, your heart rate, respiratory gases,

and perceived exertion will be monitored continuously. The incremental exercise test will begin at a workload previously determined by your training history. The workload will increase at the end of 3 minute intervals (stages) until you feel you can no longer continue.

5. After successful completion of the aerobic capacity test, you will cool down and be allowed to leave the laboratory after your heart rate is less than 100 beats per minute (bpm).
6. Your initial visit to the laboratory will last approximately 90-120 minutes.

Second, Third, Fourth, and Fifth Visits to the Laboratory:

7. On a separate day, at least 72 hours but no more than approximately 7 days, following your previous visit, you will again report to the laboratory for either a resting session (control) or to perform your first 30 minute exercise session at one of the randomly selected intensities. You will report to the laboratory for this test at the same time of day as your first visit (±30 minutes). During this testing session, you will have 3 blood draws performed and 3 saliva samples taken. All of the blood draws will be performed by a certified and experienced professional. The blood and saliva samples will be temporarily kept cool and then stored until the study is complete. After completion of this study, all of the samples will be destroyed.
8. First, you will rest in a supine (lying down, face-up) position (to control for posture) for 30 minutes, after which the pre-exercise saliva and blood samples will be taken. Approximately 1 teaspoon (3cc) of blood and saliva will be collected with each respective sample (3 total samples during each exercise session).
9. After the 30 minute resting period, the first blood and saliva sample will be taken. Next, you will be asked to put on the heart rate monitor around your chest in order to measure your HR throughout the trial. Finally, your resting heart rate and blood pressure will be taken.
10. During each of the separate exercise trials, you will be asked to warm-up for 5 minutes on the bike and stretch. You will then cycle at one of the randomly selected intensities, at approximately either 40%, 60%, or 80% of your maximal aerobic capacity. Your heart rate will be monitored every 5 minutes. You will also have wear the mouthpiece and nose-clip (as with the first session) to monitor your respiratory gases every 10 minutes. You will be able to drink water as needed. The duration of the exercise will be 30 minutes.
11. Immediately after exercise, a second blood and saliva sample will be collected. After the samples are collected, you will be allowed to actively cool down on the bike and then rest.
12. Thirty minutes after the post-exercise blood and saliva samples are taken (Step 9 above), the final blood and saliva samples will be collected, after which you will be allowed to leave the laboratory.
13. During the resting, control session, you will have 3 blood draws performed and 3 saliva samples taken, just as with the exercise sessions. However, instead of the exercise session, you will simply rest. This session serves as a control.
12. Each of these sessions should take approximately 90-120 minutes.

13. This process will be replicated for each of the sessions (40%, 60%, 80% intensity or control resting session), which will be separated by a minimum of 72 hours.

What are the possible benefits from being in this study?

- Research is designed to benefit society by gaining new knowledge.
- You may also expect to benefit by participating in this study by receiving a free physical exam, and resting ECG. You will also get to measure your aerobic capacity (maximal oxygen uptake) that can be used to aid you in any future training. The study will also provide you with your ventilatory threshold, which can also be a tool to be used in training. The serum and saliva cortisol values will also be made available to you after the study.
- This study may help to provide a better understanding of associations between serum and salivary cortisol levels in response to exercise. Both athletes and researchers could benefit from this method's non-invasive, more feasible, and practical alternative to blood sampling if the relationship between these two methodologies are understood and can be properly applied to research design and methodology

What are the possible risks or discomforts involved from being in this study?

- There is a possibility that you will experience some light-headedness, dizziness, or fainting due to maximal and exhaustive exercise. Additionally, there is a rare possibility that you will experience a heart attack or even death. However, due to your physically active status, the risk of catastrophic events is minimized. ECG and blood pressure measurements will be taken before tests to ensure proper heart functioning. The pre-screening physical exam that includes basic measurements of pulmonary, circulatory, and orthopedic function will help detect any abnormalities that may result in termination of your participation in the study. At least two individuals certified in CPR, AED, and first-aid will be present during the entire test, and a proper emergency protocol is established if there is an emergency situation.
- There is risk of bruising or infection with all blood draws. These blood draws will be administered by an experienced professional, certified in phlebotomy. Proper first-aid procedures after the blood draws will also be followed in order to minimize infection and risk of bruising.
- There is a likelihood that you will experience muscle soreness and general fatigue following exercise sessions. This discomfort will be minimized allowing at least 72 hours to pass in between sessions. Also, a proper warm-up and stretch period prior to exercise bouts will help to minimize soreness. No soreness that inhibits daily activities is likely to occur
- There is also a rare possibility that any latent illness may become evident from the exercise or blood work.
- There may be uncommon or previously unknown risks. You should report any problems to the researcher.

How will your privacy be protected?

- Every effort will be made to protect the confidentiality and privacy of the data obtained from you as a subject participating in the study in this study. You will assigned an ID number and all data entered into computer will not be identifiable by your name.
- Only the principal investigator and the faculty advisor will have access to the names and the data of each of the subjects.
- All information will be stored in locked file cabinets and secure password-protected, computer files.
- In the event that the study is published, no association will be made between reported data and your name.

You will not be identified in any report or publication about this study. Although every effort will be made to keep research records private, there may be times when federal or state law requires the disclosure of such records, including personal information. This is very unlikely, but if disclosure is ever required, University of North Carolina at Chapel Hill will take steps allowable by law to protect the privacy of personal information. In some cases, your information in this research study could be reviewed by representatives of the University, research sponsors, or government agencies for purposes such as quality control or safety.

What will happen if you are injured by this research?

All research involves a chance that something bad might happen to you. This may include the risk of personal injury. In spite of all safety measures, you might develop a reaction or injury from being in this study. If such problems occur, the researchers will help you get medical care, but any costs for the medical care will be billed to you and/or your insurance company. The University of North Carolina at Chapel Hill has not set aside funds to pay you for any such reactions or injuries, or for the related medical care. However, by signing this form, you do not give up any of your legal rights.

What if you want to stop before your part in the study is complete?

You can withdraw from this study at any time, without penalty. The investigators also have the right to stop your participation at any time. This could be because you have had an unexpected reaction, or have failed to follow instructions, or because the entire study has been stopped.

Will you receive anything for being in this study?

You will not receive anything for taking part in this study.

What if you are a UNC student?

You may choose not to be in the study or to stop being in the study before it is over at any time. This will not affect your class standing or grades at UNC-Chapel Hill. You will not be offered or receive any special consideration if you take part in this research.

What if you are a UNC employee?

Taking part in this research is not a part of your University duties, and refusing will not affect your job. You will not be offered or receive any special job-related consideration if you take part in this research.

What if you have questions about this study?
You have the right to ask, and have answered, any questions you may have about this research. If you have questions, complaints, concerns, or if a research-related injury occurs, you should contact the researchers listed on the first page of this form.

What if you have questions about your rights as a research participant?
All research on human volunteers is reviewed by a committee that works to protect your rights and welfare. If you have questions or concerns about your rights as a research subject, or if you would like to obtain information or offer input, you may contact the Institutional Review Board at 919-966-3113 or by email to IRB_subjects@unc.edu.

- -

Title of Study: The Relationship Between Plasma and Salivary Cortisol Levels in Response to Different Exercise Intensities

Principal Investigator: Mitch D. VanBruggen

Participant's Agreement:

I have read the information provided above. I have asked all the questions I have at this time. I voluntarily agree to participate in this research study.

_____ _____
Signature of Research Participant Date

_____
Printed Name of Research Participant

_____ _____
Signature of Research Team Member Obtaining Consent Date

_____
Printed Name of Research Team Member Obtaining Consent

APPENDIX D

DATA COLLECTION SHEETS

Orientation/VO_{2max} Session

Subject Name_____ Subject ID_____

Informed Consent
1. Inform participant of the experimental protocol
2. Make certain that the subject is aware of the possible risks
3. Sign informed consent

Participant Compliance Questions
1. Did subject refrain from strenuous physical activity for 24h prior to $VO_{2\ max}$ testing?
   Yes No
2. Did the subject report to the lab 4 hours post-prandial?
   Yes No
3. Did the subject take NSAIDs, consume alcohol, or caffeine 8 hours prior to testing?
   Yes No

Examinations _____
1. Medical History
2. 12 Lead ECG
3. Physical Examination

Physical Characteristics
1. Age _____ yrs
2. Height _____ cm
3. Mass _____ kg

4. *Percent Body Fat* _____%
   Skinfolds:
   a. Chest (diagonal fold midway between upper armpit & nipple) _____mm
   b. Abdominal (vertical fold; 1 inch to right of navel) _____mm
   c. Thigh (vertical fold midway between kneecap and top of thigh) _____mm

Before VO_{2max} Testing Protocol
1. Set up metabolic system (gas calibration & mouthpiece)
2. Fit electronically-braked cycle ergometer to the participant - record seat position using
   Seat height: _____ cm
3. Fit polar heart rate (HR) monitor to participant
4. Make sure polar heart rate monitor picks up signal
5. Place RPE scale near cycle ergometer/explain RPE to participant

Warm Up
1. 5 minutes of cycling at light workload
2. 5 minutes of stretching focused on the lower extremities
3. Record resting oxygen consumption to verify values fall within normal range

$VO_{2\ max}$ Protocol
1. Stage 1: 50W for 3 minutes: HR _____; RPE _____
2. Stage 2: 100W for 3 minutes: HR _____; RPE _____
3. Stage 3: 150W for 3 minutes: HR _____; RPE _____
4. Stage 4: 200W for 3 minutes: HR _____; RPE _____
5. Stage 5: 225W for 1 minute: HR _____; RPE _____
6. Stage 6: 250W for 1 minute: HR _____; RPE _____
7. Stage 7: 275W for 1 minute: HR _____; RPE _____
8. Stage 8: 300W for 1 minute: HR _____; RPE _____
9. Stage 9: 325W for 1 minute: HR _____; RPE _____
10. Stage 10: 350W for 1 minute: HR _____; RPE _____
11. Stage 11: 375W for 1 minute: HR _____; RPE _____
12. Stage 12: 400W for 1 minute: HR _____; RPE _____

13. Increase workload until volitional fatigue add more stages if necessary
14. Recovery - reduce resistance and have participant continue pedaling
15. Participant rests (supine) until HR is less than or equal to 100 bpm

Was the total test time equal to or greater than 12 minutes? Test time = _____

Criteria for valid and reliable $VO_{2\ max}$ Test
1. Was there a 150 ml/min or less increase in oxygen consumption in response to an increased workload?
Yes No
2. Did the participant have a maximal RER equal to or greater than 1.1?
RER =_____
3. Did the participant reach age-predicted maximal HR (220-age ± 5%)?
HRmax =_____
4. Did the participant have a RPE equal to or greater than 18?
RPE = _____

Recovery:
1. Reduce resistance and have participant continue pedaling
2. Allow subject to rest (supine) until HR is less than or equal to 100 bpm

Before Subjects Leave Laboratory:
1. Obtain diet 3-day diet record for analysis required before first experimental session
2. Schedule first experimental session

Experimental Exercise Sessions

Subject Name_____ Subject ID_____

Experimental sessions should start approximately one week after orientation/VO_{2max} session; spaced 72 hours apart, randomized, and all completed at about (±30 min) the same time of day.

Administer REST-Q-Sport Questionnaire for Athletes to subject

1. What was the subject's score? _____

The score must be lower than 36 (average of the midpoints of each of the 12 scales; scale ranges from 1 to 6) for the subject to proceed on that day.

Participant Compliance Questions

1. Did the subject refrain from strenuous physical activity for 24 hours prior to the experimental sessions?

Yes No

2. Did the subject report to the lab 4 hours post-prandial?

Yes No

3. Did the subject take NSAIDs, consume alcohol, or caffeine 8 hours prior to testing?

Yes No

Before Starting Exercise Protocols

1. Set up metabolic system (calibrate, mouthpiece, etc)
2. Set up blood collection supplies
3. Set up saliva collection supplies
4. Set up cycle ergometer to previously recorded seat height: _____ cm
5. Fit polar heart rate (HR) monitor to participant
6. Make sure polar heart rate monitor picks up signal
7. Place RPE scale near cycle ergometer

Pre-Exercise Rest

1. The participant will rest in the supine position for 30 minutes
2. While subject is resting, place catheter into arm.
3. After 30 minutes rest, obtain 3-mL of venous blood
4. Place blood into a sterile K^2 EDTA (purple top) Vacutainer® tube; place tube on ice
5. Obtain resting saliva sample (~0.5-1.0 mL); place saliva in polypropylene vial

If subject has trouble salivating, stimulate through chewing on paraffin film

Warm Up

1. 5 minutes of cycling at light workload (~15-20% $VO_{2\ max}$)
2. 5 minutes of stretching focused on the lower extremities
3. Record resting oxygen consumption to verify values fall within normal range

Exercise Protocol

Randomly Assigned Exercise Intensity and Corresponding Workload

____40% $VO_{2\ max}$ Workload_____W
____60% $VO_{2\ max}$ Workload_____W
____80% $VO_{2\ max}$ Workload_____W

During exercise, subjects are allowed to consumer water ad-libitum

Cycle for 30 minutes at the previously determined workload.
Minute 0: HR _____ RPE _____
Minute 5: HR _____ RPE _____
Minute 7-10 VO_2 measurement (remove mouthpiece after sampling)
Minute 10: HR _____ RPE _____
Minute 15: HR _____ RPE _____
Minute 17-20 VO_2 measurement (remove mouthpiece after sampling)
Minute 20: HR _____ RPE _____
Minute 25: HR _____ RPE _____
Minute 27-30: VO_2 measurement (remove mouthpiece after sampling)
Minute 30: HR _____ RPE _____

Immediately Post-Exercise

1. Immediately after 30 minute exercise session, obtain 3-mL of venous blood.
2. Place blood into a sterile K^2 EDTA (purple top) Vacutainer® tube; place tube on ice
3. Obtain saliva sample (~0.5-1.0 mL); place saliva in polypropylene vial
   If subject has trouble salivating, stimulate through chewing on paraffin film
4. Begin supine resting session

Resting/Recovery

1. Immediately after 30 minute supine recovery session, obtain 3-mL of venous blood.
2. Place blood into a sterile K^2 EDTA (purple top) Vacutainer® tube; place tube on ice
3. Obtain saliva sample (~0.5-1.0 mL); place saliva in polypropylene vial
   If subject has trouble salivating, stimulate through chewing on paraffin film

Before Subjects Leave Laboratory:

1. Make sure heart rate is below 100 bpm
2. Schedule next experimental session

Experimental Control (Resting) Session

Subject Name_____ Subject ID_____

Control/Resting session will be randomly assigned and may occur at any point in the timeline of the 4 separate experimental sessions. As with the exercise sessions, this session should be spaced 72 hours apart from other sessions and all completed at the same time of day (±30 min) as the other sessions

Administer REST-Q-Sport Questionnaire for Athletes to subject

1. What was the subject's score? _____

   The score must be lower than 36 (average of the midpoints of each of the 12 scales; scale ranges from 1 to 6) for the subject to proceed on that day.

Before Starting Control Session

1. Set up blood collection supplies
2. Set up saliva collection supplies

Pre-Rest Procedures

1. The participant will rest in the supine position for 30 minutes
2. While subject is resting, place catheter into arm.
3. After 30 minutes rest, obtain 3-mL of venous blood
4. Place blood into a sterile K^2 EDTA (purple top) Vacutainer® tube; place tube on ice
5. Obtain resting saliva sample (~0.5-1.0 mL); place saliva in polypropylene vial

   If subject has trouble salivating, stimulate through chewing on paraffin film

Resting Protocol

Instead of exercise, subject will rest in supine position for 30 minutes

Immediately Post-Rest

1. Immediately after 30 minute resting/control session, obtain 3-mL of venous blood.
2. Place blood into a sterile K^2 EDTA (purple top) Vacutainer® tube; place tube on ice
3. Obtain saliva sample (~0.5-1.0 mL); place saliva in polypropylene vial

   If subject has trouble salivating, stimulate through chewing on paraffin film
4. Begin supine resting position

Post-Rest Procedures

1. Immediately after 30 minute supine resting session, obtain 3-mL of venous blood.
2. Place blood into a sterile K^2 EDTA (purple top) Vacutainer® tube; place tube on ice
3. Obtain saliva sample (~0.5-1.0 mL); place saliva in polypropylene vial

   If subject has trouble salivating, stimulate through chewing on paraffin film

Before Subjects Leave Laboratory:

1. Schedule next experimental session

APPENDIX E

ASSAY INFORMATION

Serum Cortisol Assay Procedures

All components must be at room temperature (15-28º C) before use.

1. **Plain Tubes:** Label four plain (uncoated) 12 x 75 mm polypropylene tubes T (total counts) and NSB (non-specific binding) in duplicate. Because non-specific binding in the Coat-A-Count procedure is low, the NSB tubes can be omitted without compromising accuracy or quality control.
   Coated Tubes: Label twelve Cortisol Ab-Coated Tubes A (maximum binding) and B through F in duplicate. Label additional Cortisol Ab-Coated Tubes, in duplicate, for controls and patient samples.

Calibrators	µg/dL	nmol/L
A (MB)	0	0
B	1	27.6
C	5	138
D	10	276
E	20	552
F	50	1380

2. Pipet 25 µL of the zero calibrator A into the NSB and A tubes. Pipet 25 µL of each remaining calibrator, control, and patient samples into the tubes prepared. Pipet directly to the bottom. It is good practice to use a disposable-tip micropipette, changing the tip between samples, in order to avoid carryover contamination.

3. Add 1.0 mL of 125I Cortisol into every tube. Vortex. Laboratories equipped with a reliable pipettor-diluter may handle steps 2 and 3 simultaneously. No more than 10 minutes should elapse during the dispensing of the tracer. Set the T tubes aside for counting at step 6; they require no further processing.

4. Incubate for 45 minutes at 37º C. Use a water bath; neither an oven nor a heat block is suitable. Longer incubation periods will not significantly affect the assay.

5. Decant thoroughly. Removing all visible moisture will greatly enhance precision. Decant the contents of all tubes (except the T tubes) using a foam decanting rack, and allow them to drain for 2 or 3 minutes. Then strike the tubes sharply on absorbent paper to shake off all residual droplets.

6. Count for 1 minute in a gamma counter.

Salivary Cortisol Assay Procedures

1. Bring all reagents to room temperature and mix before use.

2. Prepare 1X wash buffer (and reconstitute stop solution, if appropriate).

3. Bring plate to room temperature and prepare for use with NSB wells.

4. Prepare tube with 24 mL of assay diluent for conjugate dilution, which will be made later.

5. Pipette 25 µL of standards, controls, and unknowns into appropriate wells.

6. Pipette 25 µL of assay diluent into zero and NSB wells.

7. Make final 1:1600 dilution of conjugate (15 µL into 24 mL assay diluent), mix, and immediately pipette 200 µL into each well.

8. Mix plate for 5 minutes at 500 rpm. Incubate for an additional 55 minutes at room temperature.

9. Wash plate 4 times with 1X wash buffer. Blot.

10. Add 200 µL TMB solution to each well.

11. Mix plate for 5 minutes at 500 rpm. Incubate in dark at room temperature for 25 additional minutes.

12. Add 50 µL stop solution to each well. Mix for 3 minutes at 500 rpm.

13. Wipe plate bottom clean and read within 10 minutes of adding stop.

Calculations

1. Compute the average optical density (OD) for all duplicate wells.

2. Subtract the average OD for the NSB wells from the average OD of the zero, standards, controls, and unknowns.

3. Calculate the percent bound (B/Bo) for each standard, control, and unknown by dividing the average OD (B) by the average OD for the zero (Bo).

4. Determine the concentrations of the controls and unknowns by interpolation.

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