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Hair Cortisol as a Reflection of Metabolic, Physical, and Psychological Stress in Male and Female Collegiate Athletes at Different Phases of a Competitive Season

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ABSTRACT

A competitive season represents a multi-stressor environment for athletes, posing various stressors to the body due to increased training volume and energy demands as well as increased psychological pressures. An accumulation of stress due to these factors can result in increased secretion of stress hormones in the body such as cortisol. Circulating cortisol is deposited in hair shafts, and cortisol can be extracted from hair samples and measured according to concentration. Analyzing hair cortisol concentration serves as a retrospective measure of chronic cortisol exposure, with the closest 3 cm of hair to the scalp representing the previous 3 months of hair growth and, thus, cortisol accumulated in hair. This study, as part of a larger longitudinal observational study, aims to evaluate the use of hair cortisol as a reflection of metabolic, physical, and psychological stress in male and female collegiate athletes across a competitive season. This study followed 19 athletes (9 males and 10 females) throughout a competitive season, from pre-season to peak-season to off-season. At each season phase, measurements of metabolic, physical, and psychological stress were repeated, allowing comparison across time. Such measurements included: anthropometrics and body composition via dual energy x-ray absorptiometry (DXA), resting metabolic rate (RMR) via indirect calorimetry and normalized per kg of fat free mass (FFM), energy intake via app-based diet logs, training volume via paper exercise logs, questionnaires to evaluate psychological stress, namely the Perceived Stress Scale (PSS) and Recovery-Stress Questionnaire for Athletes (RESTQ-52), and hair cortisol and cortisone. In addition, a ratio of actual to predicted RMR based on metabolically active tissue measured via DXA was calculated to represent energy status and identify potentially energy deficient individuals, representing metabolic stress, and exercise energy expenditure (EEE) was

calculated using data from the exercise logs and metabolic equivalent (MET) values from the 2024 Adult Compendium for Physical Activity, representing physical stress. We hypothesized that lower RMR/kg FFM and RMR ratio, indicating metabolic stress, higher training volume and EEE, indicating physical stress, and higher scores from the psychological stress questionnaires would be reflected by higher hair cortisol levels. The results of this study were that there were no significant relationships between these stress variables and hair cortisol over time, suggesting that hair cortisol does not serve as a reflection of metabolic, physical, or psychological stress across a competitive season. The only significant relationship found was a positive relationship between RMR/kg FFM and RMR ratio and hair cortisol ($r=0.6$, $p=0.018$; $r=0.646$, $p=0.009$), as well as cortisol/cortisone ratio ($r=0.643$, $p=0.01$; $r=0.564$, $p=0.028$), within the off-season, possibly explained by increased training volume during the peak-season increasing RMR and stress, as reflected by hair cortisol. Although this study did not produce the results as expected by our hypothesis, there were several limitations, such as a small sample size and variations in the amount of time in between season phases for athletes in different sports, that when addressed in future studies could produce more accurate and generalizable results, presenting a clearer picture of the effects of different types of stress on cortisol exposure.

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Chapter 1

Literature Review

Introduction

Stress can be broadly defined as the body's response to any challenge or disturbance, and it can have physical, mental, and emotional components (Ramanathan and Desrouleaux, 2022). In the context of biology, stress activates the nervous system, eliciting neuroendocrine and behavioral changes to promote survival (Ramanathan and Desrouleaux, 2022). Stressors can come in a variety of forms, including physical stressors such as exercise (Skoluda et al., 2012), sleep disruption due to shift work (Manenschijn et al., 2011), chronic pain (Van Uum et al., 2007), and pregnancy (Kirschbaum et al., 2009), or psychological stressors, including major life stressors (Karlén et al., 2011), depression (Dettenborn et al., 2012), post-traumatic stress (Steudte et al., 2011), and other mental health disorders. College athletes undergo both physical and psychological stress due to the demands of their training (Skoluda et al., 2012), academics (Egan 2019), and other social and life stressors (Ströhle 2019), thereby increasing stress hormones such as cortisol. In several studies, chronically elevated stress has been found to be reflected physiologically, i.e., as higher circulating cortisol levels resulting in more cortisol deposited in hair segments of athletes compared to non-athlete controls (Skoluda et al., 2012; Gerber et al., 2013).

A metabolic stressor highly prevalent in athletes is energy deficiency (Marzuki et al., 2023). Resulting from higher energy expenditure relative to energy intake, many athletes may be

energy deficient (Marzuki et al., 2023), meaning that they do not consume enough calories to meet their body's energy needs (Nattiv et al., 2007; De Souza et al., 2014). Low energy availability, defined as dietary energy intake minus exercise energy expenditure and normalized to fat free mass (Nattiv et al., 2007), is caused by clinical eating disorders or subclinical disordered eating behaviors, and in some cases, inadvertently undereating, (Nattiv et al., 2007) and can have negative effects on health and performance (Nattiv et al., 2007). Several studies, mainly in anorexia nervosa patients, indicate that energy deficient individuals exhibit higher circulating cortisol as a metabolic counterregulatory mechanism, as indicated by higher 12-hour and 24-hour serum and urine cortisol measures (Thavaraputta et al., 2023). However, current research regarding hair cortisol in anorexia nervosa patients is limited and has inconclusive results as to whether this chronic hypercortisolism in these individuals is reflected in hair cortisol; one study found no significant difference in anorexics vs. non-anorexic controls (Ritschel et al., 2018), while another study actually found lower hair cortisol in anorexics compared to non-anorexic controls (Föcker et al., 2016). In athletes specifically, studies demonstrate an increase in 24-hour area under the curve (AUC) serum cortisol associated with energy deficiency and consequent weight loss (Ruffing et al., 2022), but no studies to date have demonstrated elevated hair cortisol in athletes due to energy deficiency.

This paper aims to explore the relationship between energy deficiency (Marzuki et al., 2023) and physical (Skoluda et al., 2012) and psychological stress (Ströhle 2019), in athletes by examining hair cortisol as an indication of elevated hypothalamic-pituitary-adrenal axis activity. The purpose of this project is 1) to determine if hair cortisol is reflective of metabolic, physical, and psychological stress, and 2) to determine which, if any, of these stressors is most strongly reflected in hair cortisol in male and female collegiate athletes at different phases of a

competitive season. By comparing hair cortisol measures to psychometric indices of stress, laboratory measures of energy deficiency, and records of training volume, and by testing whether hair cortisol levels increase in response to exposure to a competitive season, we aim to establish a model using a non-invasive, quick and easily accessible biological marker to determine the overall stress of athletic competition. Therefore, this model can be further used to answer questions that build on the idea that a competitive season represents a multi-stressor environment.

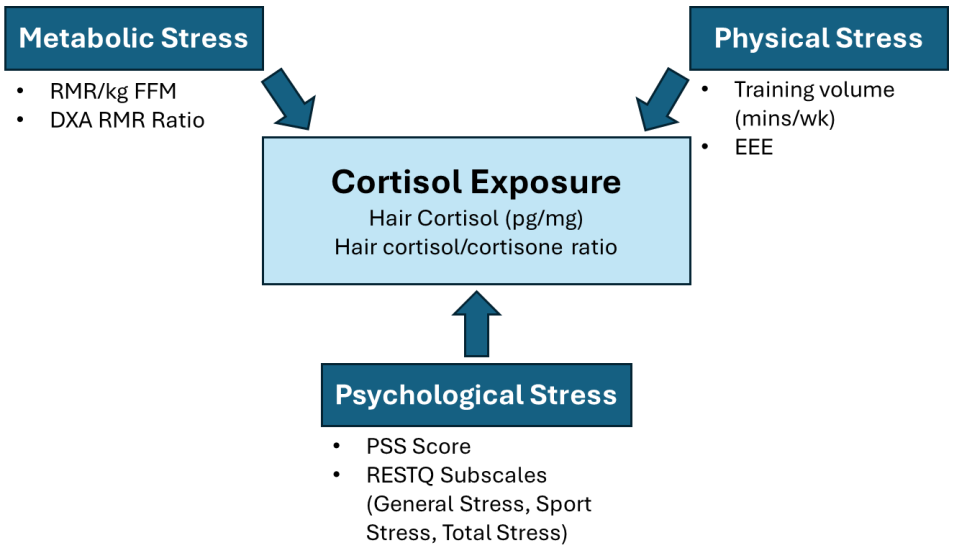

Figure 1 Schematic for analysis of cortisol exposure due to metabolic, physical, and psychological stress.

Energy Deficiency in Athletes

The female and male athlete triad

Low energy availability, characterized by not consuming enough calories to meet energy needs due to exercise (Nattiv et al., 2007), leads to metabolic suppression to conserve energy for

vital functions at the expense of "non-vital" functions such as reproduction and growth (Wade et al., 1996). This forms the basis of the medical condition called the athlete triad (Nattiv et al., 2007; De Souza, et al., 2014; Nattiv et al., 2021), affecting both female and male athletes and exercising individuals. The athlete triad is a well-defined and supported condition characterized by interrelationships between energy availability, reproduction, and bone health, where varying degrees of low energy availability, with or without disordered eating, result in suppressed reproductive function and eventually decreased bone health (Nattiv et al., 2021). First named the female athlete triad (Nattiv et al., 2007), most research into this condition has been conducted in female subjects only and examined effects on menstrual function and ovarian hormones; however, in recent years, research has expanded to include males, exhibiting similar reproductive disturbances. Thus, the Female and Male Athlete Triad Coalition issued a consensus statement in 2021 defining the male athlete triad (Nattiv et al., 2021).

When energy intake is chronically inadequate to meet energetic demands, the hypothalamus goes into "survival mode," shunting energy away from reproductive processes (Wade et al., 1996) and leading to suppression of the hypothalamic-pituitary-gonadal (HPG) axis (Nattiv et al., 2021) – that is, the concert between the hypothalamus and pituitary gland in the brain, and the gonads (ovaries in women and testes in men). In this condition of "hypogonadotropic hypogonadism," the hypothalamus reduces its secretion of gonadotropin-releasing hormone (GnRH) in both men and women, causing downstream suppression of pituitary hormones, mainly luteinizing hormone (LH), and gonadal hormones (estrogen and testosterone) (Nattiv et al., 2021). As a result, varying degrees of menstrual dysfunction occurs in women (Nattiv et al., 2007) and abnormal sperm and decreased libido occur in men (Nattiv et al., 2021).

In addition, energy deficient individuals exhibit growth hormone resistance, whereby secretion of growth hormone from the pituitary gland is increased but resulting secretion of insulin-like growth factor-1 (IGF-1) is decreased as a protective mechanism to conserve energy and shunt away from growth (Schorr and Miller, 2016). Consequently, bone loss occurs from abnormal bone turnover, increasing risk for bone stress injuries (stress fractures) in athletes (Rizzone et al., 2017). This can potentially cause long-term consequences, as bone mass typically peaks in the late adolescent and early adult years and then declines throughout adulthood (Weaver et al., 2016). Therefore, decreased bone mass during these critical years greatly increases the risk of osteopenia (moderate bone loss) or osteoporosis (severe bone loss) later in life, especially as estrogen declines postmenopausally (Nattiv et al., 2007).

The HPA axis and energy deficiency

The interplay between the hypothalamus, pituitary gland, and adrenal glands, termed the hypothalamic-pituitary-adrenal (HPA) axis, controls stress responses in the body (Fulford et al., 2005). When the brain recognizes a stressor, the hypothalamus responds by secreting hypothalamic-releasing factors, corticotrophin-releasing factor (CRF) and arginine vasopressin (AVP), which stimulate the pituitary gland to release adrenocorticotrophic hormone (ACTH) into the bloodstream (Fulford et al., 2005). ACTH then binds to receptors on the adrenal cortex of adrenal glands and catalyzes a conversion of cholesterol esters into free cholesterol and subsequently cortisol, the glucocorticoid present in humans (Fulford et al., 2005). Only a small amount of cortisol is stored in the adrenal glands; the rest is released into the bloodstream as a rapid response to stress (Fulford et al., 2005). On average, humans have cortisol secretion rates

of about 8-25mg/day, but in conditions of increased ACTH, likely during a stressful event, cortisol release can increase to rates up to 200-250mg/day (Fulford et al., 2005).

Glucocorticoids, namely cortisol, respond to both physical and psychological stress by regulating glucose homeostasis and glycogen metabolism, with the goal of preserving blood glucose for the brain so that it can function optimally (Kuo et al., 2015). Cortisol does this both by increasing gluconeogenesis in the liver and by acting as an insulin antagonist, decreasing glucose uptake and utilization by skeletal muscle and white adipose tissue (Kuo et al., 2015). This creates a temporary hyperglycemic state, mobilizing glucose into the bloodstream so that it can fuel the brain to respond to a stressor, especially in times of fasting or starvation (Kuo et al., 2015). Thus, energy deficiency poses a physiological stress to the body, as the HPA axis must be activated to increase cortisol secretion to maintain euglycemia (normal blood sugar levels) as hypoglycemia (low blood sugar) can result from insufficient caloric intake (Kuo et al., 2015). Literature has established the presence of hypercortisolism in anorexia nervosa patients using acute cortisol measures such as 24h urine, blood, and saliva (Föcker et al., 2016).

Activation of the HPA axis occurs during exercise, a physical stressor, as well (Hill et al., 2008). In a study of ACTH and cortisol response to exercise intensity, both increased pre- to post-exercise at 60% and 80% of VO2max during moderate and high-intensity exercise, respectively (Hill et al., 2008). After cortisol is released during exercise, it is taken up by tissues such as skeletal muscle and adipose tissue and used to carry out physiological processes needed to perform the exercise and recover, such as breaking down proteins in the muscles or triglycerides in adipose tissue (Hill et al., 2008).

Stress in Athletes

Athletes demonstrate higher stress than non-athletes

Due to an increased physical and psychological demand or pressure from training and competition, athletes may experience higher overall stress than non-athletes (Skoluda et al., 2012; Ströhle 2019; Lopes Dos Santos, 2020). Specifically, athletes are more likely to experience psychological stress from things like negative athletic cultures (Wiese-Bjornstal 2010), overtraining (Gottschall et al., 2020), negative body image and eating disorders (Egan 2019), and environmental factors that impact mental health such as hazing and transition from sport (Chang et al., 2020). High stress has been supported by multiple studies comparing hair and other methods of cortisol analysis in athletes of varying training regimens to estimate stress responses (Skoluda et al., 2012; Gerber et al., 2013). One study compared cortisol measured in hair samples in endurance athletes and non-endurance-athlete age-matched controls and found a significantly higher cortisol concentration (46%) in endurance athletes than non-athlete controls, and a dose-response relationship between training volume and hair cortisol concentration (km run per week or hours per week) (Skoluda et al., 2012). These findings suggest endurance athletes are exposed to higher glucocorticoid levels over prolonged periods of time than non-athletes which could possibly lead to increased risk for disease development (Skoluda et al., 2012).

Another study objectively assessed the effect of physical activity level, determined by accelerometer data, on hair cortisol concentration over a three-month period (represented by a 3 cm hair segment) in young adult athletes, finding a significant positive correlation between hair

cortisol and vigorous physical activity, but not moderate physical activity (Gerber et al., 2013). In addition, there was no correlation between perceived stress and hair cortisol, indicating that the physical stress of exercise may have a greater influence on cortisol levels than psychological stress (Gerber et al., 2013). These findings support previous studies (Skoluda et al., 2012) that demonstrate a dose-response relationship between training volume and hair cortisol in endurance athletes. Therefore, it can be inferred that since vigorous physical activity leads to an increase in salivary and plasma cortisol concentrations, which are acute measurements, overall heightened hair cortisol concentration, a more chronic measurement, reflects chronically high exercise-induced HPA activation (Gerber et al., 2013).

The implications of prolonged high stress in athletes are numerous, both physically and psychologically. Overreaching, or experiencing overload to the neuroendocrine system due to extreme demands of high-volume training, can occur, indicated by high salivary cortisol levels at rest and decreased cortisol response to exercise (Gottschall et al., 2020). This dysregulation can lead to many negative consequences for athletes such as decreased performance, fatigue, sleep disturbances, changes to resting and exercise heart rate, and depression (Gottschall et al., 2020). Crucial for athletes, bone health can be compromised by high cortisol because of reduced osteoblast, or bone-building, activity and decreased calcium absorption (Thavaraputta et al., 2023), making bone-stress injuries more likely. In addition, although somewhat moderated by the positive effects of exercise (Skoluda et al., 2011), chronically elevated cortisol has been linked to increased risk for cardiovascular disease and metabolic disorders such as diabetes (Iob and Steptoe, 2019). Psychologically, high stress can interfere with sports performance and increase risk for mental health disorders such as depression and anxiety (Putukian and Yeates, 2023).

Measures of psychological stress in athletes

Common measures of psychological stress used by researchers include the Perceived-Stress Scale (PSS) (Cohen et al., 1983), the Recovery-Stress Questionnaire (REST-Q) (Kallus and Kellman, 2016), the Type-D personality scale (DS-14) (Denollet 2005), the General Health Questionnaire (GHQ-12) (Goldberg and Hillier, 1979), General Self-Efficacy Scale (Schwarzer and Jerusalem, 1995), and the Trier Inventory for Chronic Stress (Schulz and Schlotz, 1999). Many studies assessing the relationship between psychological stress and hair cortisol use the PSS, in particular, to assess stress levels (Staufenbiel et al., 2013).

Measures of physiological stress in athletes

For measuring physiological stress, research studies utilize many different biochemical tests, the majority of which measure cortisol in different methods. Among the most common include morning serum cortisol, 12-hour and 24-hour pooled serum cortisol, 24-hour urine cortisol, salivary and late-night salivary cortisol, cerebrospinal fluid sample, DHEA-S, low-dose dexamethasone suppression test, and hair sampling (Thavaraputta et al., 2023). Notably, cortisol has a circadian variation with low levels during sleep and high levels upon awakening, especially within the first 30 minutes, termed the cortisol awakening response (CAR) (Dahlgren et al., 2009), so serum cortisol is often tested in the morning to reflect this response. Most of these methods measure short-term cortisol levels, either by a single timepoint or for a single day and incur limitations due to the diurnal variation of cortisol release and protein binding (naturally higher or lower cortisol at different times of day) (Thavaraputta et al., 2023; Dahlgren at al., 2009). However, hair samples indicate average cortisol levels over a greater period (Greff et al.,

2018), in a retrospective manner. Therefore, exploration of hair cortisol in athletes is warranted as a longitudinal measure of stress as compared to the aforementioned single-timepoint measures commonly used in athletes.

The acute HPA-activation response to exercise has been quantified in many studies by collecting salivary, blood, or urine samples during and/or immediately following exhaustive exercise, usually aerobic and endurance, and measuring cortisol in the samples (Skoluda et al., 2012). These studies have demonstrated increased cortisol levels as a response to exercise, with a positive correlation between cortisol and intensity or duration of exercise (Skoluda et al., 2012). While blood, urine, and salivary measurements of cortisol are extremely useful in determining acute cortisol responses to this physical stressor, they only represent brief time points and do not provide a picture of long-term HPA activation resulting from cumulative stress exposure (i.e., in athletes who regularly train and compete).

To estimate the effects of cumulative and long-term stress due to repetitive exercise exposure in athletes over time, it is necessary to utilize a chronic biological measurement, i.e. hair cortisol. Few studies have been performed to measure hair cortisol in athletes, but the general method of doing so has been to take cortisol measurements at different timepoints before and after a training period or competitive season in order to compare baseline circulating cortisol levels with those resulting from long-term stress exposure (Skoluda et al., 2012, Gerber et al., 2013). The Kirschbaum protocol is then used to sample hair, process and wash samples, and extract cortisol for quantitative analysis (Kirschbaum et al., 2009).

Hair Cortisol

Hair cortisol as a measure of chronic stress

Cortisol and other small, hydrophobic metabolites consistently in circulation are incorporated into hair shafts, providing a unique opportunity to collect and measure their concentration in a hair sample (Greff et al., 2018). Therefore, hair cortisol is a promising biomarker for analysis of endogenous cortisol levels for clinical purposes and in research, and it has potential to advance diagnosis and patient care (Greff et al., 2018). Since cortisol fluctuates throughout the day due to circadian rhythm, activity, and acute stressors, taking a single timepoint measurement does not give an accurate depiction of overall stress indicated by cortisol levels in the body (Russell et al., 2012). As opposed to single-timepoint measures of cortisol levels, such as in serum, saliva, or urine, hair provides a measurement of cortisol circulating through the body over a longer time period (Greff et al., 2018). Hair samples can also easily be stored at room temperature for extended periods, as opposed to the extensive storage, freezing, and thawing process of urine or blood samples (Greff et al., 2018).

The literature demonstrates that each centimeter of hair closest to the scalp represents one month's worth of cortisol incorporated into hair, with the first 3 cm being the most accurate due to a wash-out effect further along the hair shaft (Stalder et al., 2017). Kirschbaum et al. (2009) conducted the first study to validate using hair analysis as a retrospective calendar of endogenous cortisol secretion and used pregnancy as a model of physiological stress, as studies have shown a significant increase in cortisol during the third trimester. The main findings were that the most proximal 3-cm hair segment, reflecting the third trimester of pregnancy, had significantly higher

cortisol concentration than more distal segments, and that cortisol measured in hair can be a valid reflection of increased cortisol production for a period of up to 6 months (Kirschbaum et al., 2009).

Effects of physiological vs. psychological stress on hair cortisol concentration

Several studies have been conducted to compare the effects of psychological stress and physiological stress on chronic cortisol exposure, represented in hair samples. Currently, the literature supports that physiological stressors have demonstrated a much stronger correlation with hair cortisol than psychological (Dettenborn et al., 2010; Staufenbiel et al., 2013; Gerber et al., 2013; Skoluda et al., 2012). One study conducted by Dettenborn et al. in 2010 found that unemployed individuals had higher scores on psychological stress measures and higher cortisol content in the first and second hair segments than employed (Dettenborn et al., 2010). However, hair cortisol content was unrelated to any of the psychosocial variables studied, thus termed "lack of psychoendocrine covariance." (Dettenborn et al., 2010). These findings are consistent with similar studies and support that hair cortisol concentration may be a useful biomarker for stress because it reflects cortisol rates over long time periods to represent the physiological burden of stress.

Relationships between stress, energy deficiency, and hair cortisol

As established earlier in this paper, athletes often exhibit higher stress than non-athletes due to physical and psychological demands of their training and lifestyles (Skoluda et al., 2012;

Ströhle 2019; Lopes Dos Santos, 2020). In addition, energy deficiency is highly prevalent in athletes, due to both an increased likelihood of eating disorders (Chang et al., 2020) and inadvertent undereating for increased exercise energy expenditure. In athletes, where energy deficiency is found, stress is often also found, and vice versa. There are several proposed links between these two conditions, but not one causal direction; for example, higher social stress may increase likelihood of developing an eating disorder (Monteleone et al. 2020), or lack of energy when an eating disorder is present may increase physiological stress responses (Kuo et al., 2015). Across competitive sports seasons and chronically, high training loads, psychological stress, and energy deficiency can pose significant stressors, potentially correlating with a chronically elevated level of cortisol. Currently, literature is extremely limited in relating energy deficiency to hair cortisol. One study demonstrated lower hair cortisol in anorexia nervosa patients, but this may have been confounded by endocrine alterations due to anorexia pathology that affected hair growth (Föcker et al., 2016), and another study demonstrated no significant difference in hair cortisol in anorexia patients vs. non-anorexic controls (Ritschel et al., 2018). To our knowledge, there is no research available investigating the link between energy deficiency and hair cortisol specifically in athletes. Therefore, it is imperative that these links be investigated further by analyzing hair cortisol samples in athletes, a measure of chronic stress, to determine how these stressors affect the body in the long-term.

Measuring Energy Deficiency and Hair Cortisol

How physical activity and energy deficiency are measured in the lab

Laboratory settings often assess physical activity using a wearable heart rate monitor, which captures intensity of exercise, as well as self-reported exercise and activity logs (Ainsworth et al., 2014). This data can further be analyzed to estimate exercise energy expenditure (EEE), which factors into an individual's total daily caloric expenditure. To determine total energy expenditure, labs must also measure resting energy expenditure, or REE (Blasco Redondo 2015) (also called resting metabolic rate, or RMR). To do so, indirect calorimetry is most commonly used by measuring O_2 and CO_2 inflow/outflow in a resting, fasted state under a ventilated hood to calculate calories expended at rest (Strock et al., 2020). Then, the sum of EEE and REE provide an approximate estimate of total daily energy expenditure (Blasco Redondo 2015).

To assess energy intake, researchers commonly administer self-report diet logs to subjects (Foster et al., 2019). However, this self-report method is often prone to error (Foster et al., 2019; De Souza et al., 2019), as individuals must exactly track their food intake, which has significant room for inaccurate results due to under- or over-reporting. Despite being prone to error, however, online self-report diet logs are a commonly used assessment tool, demonstrating good relative validity (Teixeira et al., 2018; Albar et al., 2016) and providing a more timely and cost-effective method of assessment than interviewing and manual calculations (Foster et al, 2019). This method of estimating energy intake has been used to calculate energy availability by comparing energy intake to exercise energy expenditure.

A study conducted by Strock et al. in 2020 demonstrated a more accurate method of estimating chronic energy deficiency by using an RMR ratio, or the ratio of an individual's actual measured RMR to their predicted RMR based on their anthropometric measurements (Strock et al., 2020). If the ratio falls below a certain threshold based on three different equations, it indicates likely energy deficiency in an individual (Strock et al., 2020). In this study, anthropometric measurements and energy expenditure were evaluated using dual-energy x-ray absorptiometry (DXA) and indirect calorimetry, and then Harris-Benedict, DXA, and Cunningham equations were used to predict RMR and RMR ratio (Strock et al., 2020). This study found that a 0.90 ratio threshold yielded highest sensitivity for Cunningham_{1980} and Harris-Benedict methods, but a 0.94 ratio threshold was best for DXA-derived RMR (Strock et al., 2020). A positive correlation between RMR ratio and TT3 (triiodothyronine) levels in subjects validated these values; those with a low RMR ratio, often those who are energy deficient and exhibit menstrual disturbances, also tend to exhibit lower TT3 levels due to suppressed metabolism (Strock et al., 2020).

Method of hair cortisol measurement

The measurement and analysis of hair cortisol has become a relatively standardized process used by many studies citing to the protocol established by Kirschbaum et al., 2009. This process involves hair sample collection, wash, extraction, and assay (Kirschbaum et al., 2009). According to the Kirschbaum protocol, researchers take a hair sample from the posterior vertex of the head, as close to the scalp as possible for the most accurate measurement (Kirschbaum et al., 2009). Then, the sample is finely minced with scissors or a ball mill, incubated in solvent to

wash, and evaporated to dryness. The sample is then reconstituted in solution such as a phosphate buffered saline to extract the cortisol. Finally, the cortisol assay is run using either ELISA, RIA, or LC-MS/MS (Russell et al., 2012) and concentration is analyzed.

Clinical Applications

Clinical applications of hair cortisol analysis

Researchers and physicians have used hair cortisol concentration analysis clinically to diagnose and retrospectively track development of certain disorders characterized by an alteration of stress hormone secretion (Greff et al., 2019). Cushing's disease can develop over time and is characterized by an overproduction of cortisol and consequent cardiometabolic alterations (Greff et al., 2019). Hair cortisol can be used to retrospectively track development of this condition and response to treatment, as well as detect cyclical Cushing's, a rare condition with periods of high and low cortisol circulation (Greff et al., 2019). Similarly, adrenal insufficiency, or low production of adrenal hormones, is often caused by an autoimmune response; physicians can retrospectively track it to follow development, progression, and response to treatment (Greff et al., 2019). Other clinical applications of hair cortisol analysis include depression, cardiovascular disease, recent myocardial infarction (heart attack), diabetes mellitus, obesity, severe chronic pain, post-traumatic stress disorder (PTSD), and endometriosis (Greff et al., 2019; Wester and Rossum, 2015).

Analyzing hair cortisol elucidates the role of physiological and psychological processes on the long-term activity of the HPA axis. This can help to develop preventative and therapeutic

interventions to reduce physiological mechanisms of allostatic load as a result of such stressors. Clinical settings can use hair cortisol concentration analysis to track baseline alterations, monitor interventions, identify prodromes to certain illnesses, and find comorbidities in patients with HPA axis dysregulation (Staufenbiel et al., 2013).

Elevated hair cortisol has negative health effects

Chronically elevated cortisol levels circulating in the body can have many negative effects on health. High cortisol has been linked to cardiometabolic disease factors, including hypertension, obesity, hyperglycemia, hyperlipidemia, and insulin resistance (Whitworth et al., 2005). When cortisol is elevated, it promotes gluconeogenesis and suppresses glucose uptake, suppresses insulin and contributes to insulin resistance and inflammation that underlie Type 2 Diabetes (Sharma and Singh, 2020). Supported by a study conducted by Wester and Rossum in 2015, researchers found a significant positive correlation between hair cortisol concentration and body mass index (BMI) and waist-hip-ratio (WHR) (Wester and Rossum, 2015). High cortisol can contribute to bone loss as well; it is known to diminish osteoblast proliferation, decreasing bone formation, decrease synthesis of IGF-1 in osteoblasts, reduce calcium absorption in the gut, and increase urine calcium excretion (Thavaraputta et al., 2023). Studies on anorexia nervosa patients have found significant bone loss, likely due to elevated cortisol levels as a response to low energy availability (Thavaraputta et al., 2023). Other adverse health effects of elevated cortisol include increased inflammation, which can suppress immune function (Skoluda et al., 2012) and reproductive suppression and reduced fertility due to suppression of the hypothalamic-pituitary-gonadal (HPG) axis (Russell et al., 2012).

Future Directions

Limited research is available regarding athletes and hair cortisol. However, research demonstrates that hair cortisol may be a promising biomarker for studying long-term stress exposure, a phenomenon tied closely with the physical and psychological stressors of athletic training and competition across a season (Lopes Dos Santos et al., 2020). Stress and energy deficiency are difficult to assess, especially in athletes in the midst of a competitive season who do not have the time to dedicate to research lab procedures. For this reason, we aim to establish hair cortisol as a useful biological marker of overall stress of athletic competition, investigating the specific relationships between cortisol and psychometric measures, training load, and evidence of energy deficiency in college-aged athletes.

If relationships between energy deficiency and hair cortisol concentration are found, this method could provide a less time-consuming and less invasive method to detect stress on the body related to energy deficiency than using more traditional methods of measuring caloric intake and expenditure in a lab setting. This would allow for more field-based screening, increasing availability to athletes who may not have access to a lab or time to undergo more extensive procedures. Increasing access to such screening could help prevent adverse health problems such as reproductive suppression, bone loss, and injuries which commonly occur in athletes as a result of inadequate fueling and the cortisol stress response. This is especially crucial for young athletes, as some of these conditions can affect their long-term health; in some aspects such as bone density loss, it is unknown whether the consequences of energy deficiency can be reversed (De Souza et al., 2022). In addition, screening for energy deficiency can help to

detect the presence of and raise awareness of disordered eating behaviors in athletes, allowing intervention for improved physical and mental health.

Chapter 2

Methods

Overview

The goal of this study is to assess the relationships between hair cortisol concentration and measures of metabolic, physical, and psychological stress. This study is part of a larger longitudinal observational study investigating psychobiological factors that impact the development and manifestation of eating disorder (ED) pathology in elite male and female athletes across a competitive season, with measurements occurring at pre-season, peak-season and off-season. Measurements for the present study included assessment of psychological stress via stress questionnaires, physical stress via indicators of training load, and metabolic stress via measures of body composition, metabolism, and energy status, as well as cortisol extracted from hair samples, repeated across the season. Other variables measured included cognitive variables associated with ED (cognitive flexibility, response inhibition, and reward delay), ED pathology, and gonadal hormone levels; however, these variables are beyond the scope of the present study and excluded from the present analysis.

Study Design

Recognizing that a competitive sport season serves as a natural multifactorial stressor in collegiate athletes, this study utilized a longitudinal, observational study design, monitoring collegiate athletes across a full NCAA season, spanning pre-, peak-, and off-season. This study, titled Psychological and Biological Determinants of Eating Disorder Pathology in Endurance and

Aesthetic Athletes, or The Athlete Study, was approved by the Pennsylvania State University Institutional Review Board and was funded by the Pennsylvania State University Social Science Research (Study #00018984). We acknowledge that the findings and conclusions do not necessarily reflect the view of the funding agency.

The participants of the present study included a subset of 19 collegiate athletes (n=9 males, n=10 females) from endurance/leanness sports (cross country/distance running, swimming, triathlon), aesthetic sports (gymnastics, diving, cheerleading/dance), team/intermittent sports (lacrosse, rugby), and weight-class sports (powerlifting). After obtaining written informed consent, participants began the study procedures. Data was collected during three periods during the main phases of the collegiate competitive season, based around the participants' specific competitive sport season: a pre-season period, a peak season period, and off-season period. The pre-season timepoint was defined as the onset of the competitive season, in which athletes were ramping up training volume and increasing training frequency. The peak-season was defined as the period during the competitive season in which athletes experienced their heaviest training volume, participated in competitions/meets/races (associated with the NCAA/competitive season), and had the highest competition frequency. Off-season measurements occurred during participants' period of active resting, lacking active competitions and possibly including post-season training, and/or at least 1 month following the final competition during peak-season. Measurements for each timepoint occurred at least 1 month apart, depending on an athlete's sport season schedule.

During each period of data collection, participants visited the lab for a resting metabolic rate (RMR) test, a blood draw for metabolic hormones, a dual-energy x-ray absorptiometry (DXA) scan for body composition, questionnaires for psychological stress assessments, a hair

collection to measure cortisol concentration, and a VO2max test for assessment of fitness. They were asked to record their dietary intake for 3 days (2 weekdays and 1 weekend day) and record their exercise and activity over a 7-day period, using a wearable heart rate monitor and paper logs. The purpose of this analysis was to identify the potential utility of hair cortisol as a biological marker of metabolic, physical, and psychological stress.

Athlete Recruitment

Prospective participants in this study were recruited via flyers posted on campus and in the local community, emails sent to college department listservs through department administrators, and through the Penn State university website for research volunteers. In addition, the study team provided flyers to coaching staff of athletic teams of interest, made announcements in classrooms to the athletic teams, and utilized StudyFinder. The coaches were not involved in the recruitment process, and interest or lack of interest in the study did not impact team membership in any way. For a team announcement, the study team informed the coach of study details and asked them to step out of the room during the recruitment process, then made a brief announcement to the team and handed out informational flyers with a QR code for mobile signup. The research study team utilized a verbal script for recruitment and emphasized that participation is completely voluntary and that their position on their athletic team and relationship with coaches or teammates would not be altered based on their study participation.

Interested prospective participants then contacted study staff via phone or email to schedule a time to conduct a preliminary phone screen to determine eligibility. Eligible participants had to be either A) a member of a Penn State Division I NCAA sports team, B) Penn

State-affiliated competitive club team, or C) a Penn State student currently participating and competing in a privately funded community sport team inclusive of endurance and/or aesthetic and/or leanness sports with a defined 24-36wk competitive season. Inclusion criteria included i) age 18-25 years, ii) BMI of 16.5-32 kg/m2, iii) generally good health with no serious or chronic health conditions, iv) training without any modifications that reduce participation, v) no history of psychosis or active eating disorder, vi) no apparent metabolic, endocrine, or musculoskeletal disease, vii) non-smoking, viii) not pregnant or lactating (females), and ix) not taking any hormonal medication (currently or for the past 6 months); including testosterone replacement therapy, methyltestosterone, DHEA, finasteride for male participants; including hormonal contraception for female participants. Exclusion criteria identified during the screening process included active substance use disorder, procedures using contrast material within the past 7 days (including X-rays, MRI, CT scans, barium studies, nuclear medicine exams), prostheses, vasectomy or orchidectomy (male) or hysterectomy or oophorectomy (female), unwilling to adhere to study protocol, or does not speak English or are unable to give consent.

Testing Procedures

After obtaining written informed consent, participants received written and verbal instructions for all procedures and received a "study map," visually describing the flow of the study. This study involved three assessment periods: pre-season, peak-season, and off-season. During each of the measurement periods (pre-, peak-, off-season), participants had to visit the lab for testing approximately three times; therefore, participants had about eleven visits total,

including one for general screening and informed consent and one for study completion after all three assessment periods were completed.

During each season phase, the first lab visit consisted of taking anthropometric measurements of height and weight, REDCap questionnaires to assess general health (Health, Exercise, and Nutrition Survey; Wellness Questionnaire), eating disorder (ED) psychopathology (Eating Disorder Inventory-3 (EDI-3); Three Factor Eating Questionnaire-Revised 21 (TFEQ-21); Eating Disorder Examination; Drive for Muscularity Scale (DMS); Drive for Leanness Scale (DML)], and stress [Perceived Stress Scale (PSS); Recovery-Stress Questionnaire for Athletes (RESTQ-52)], and distributing instructions for appointment 2 procedures. The second visit consisted of repeated anthropometric measurements, assessment of resting metabolic rate (RMR), blood draw for serum TT3, assessment of body composition via dual-energy X-ray absorptiometry (DXA), distribution of polar watch and heart rate monitor for assessing activity and instructions for use, as well as 7-day exercise and activity logs and instructions for a 3-day diet log using the MyFitnessPal application. The third visit consisted of repeated anthropometric measurements, a hair sample to assess chronic stress via hair cortisol, and assessment of fitness and performance via a countermovement jump test (CMJ) and VO2max test.

Anthropometrics and Body Composition

During the first laboratory visit of each study phase, height was measured using a stadiometer in cm to the nearest 0.1 cm, and weight was measured using a physician's scale (Seca Model 770; Hamburg, Germany) in kg to the nearest 0.01 kg. These measures were used to

calculate body mass index (BMI) (kg/m2), and weight was remeasured during each subsequent lab visit to ensure accuracy in testing outcomes.

Body composition was assessed during each phase of the study using dual-x-ray absorptiometry (DXA) (Hologic Horizon-W, Model 201331) performed by an International Society of Clinical Densitometry-certified technician. Subjects underwent a whole body DXA scan to determine body composition (lean mass, fat free mass, fat mass, and body fat percentage) and were required to lie still on an un-enclosed padded bed for the approximate five-minute duration, wearing clothes not containing any metal. This procedure was repeated across all season phases. DXA data was later used to predict resting metabolic rate (RMR) and calculate DXA-derived RMR ratio.

Energy Intake

Participants were given verbal and written instructions on how to complete a 3-day diet log for each phase of the study, using the MyFitnessPal (MFP) application (Under Armour, Baltimore, MD) to record all supplements, food and beverages they consumed for 2 weekdays, and 1 weekend day. The research team set the caloric intake goals on each MFP account to an arbitrary high number, such as 4000 kcal, to eliminate any potential incentive to try to "reach" a daily goal set by the app. Participants were instructed to record all food and beverage intake into the app in real time (as they consumed it) for the three total days. Energy intake (EI) was determined as kcal/day, and dietary intake data was used to calculate energy availability.

Exercise and activity assessment

Participants were given a heart rate monitor and Polar watch to track exercise for a week and instructed to complete a 7-day exercise log and activity log. Verbal and written instructions were given to participants for completing and using these items. Participants wore the Polar watch continuously during the data collection week throughout all seven days, including during sleep, and wore the accompanying heart rate monitor, a strap around the chest, during purposeful physical activity to document heart rate response, energy use (in kilocalories), and exercise duration. Participants completed the 7-day exercise log to verify all exercise activity recorded by the Polar device and to verify training phase-specific characteristics (training duration/volume, types of activities). Non-purposeful physical activity and sleep time was recorded on the 7-day activity log. In addition, participants recorded when they put on or took off the Polar watch each day, what activities they had done, and sleep and wake times each day. Exercise data was then used to calculate approximate exercise energy expenditure (EEE) using the updated 2024 Adult Compendium of Physical Activities (Herrmann et al., 2024). Specifically, activity types listed on the participants' exercise logs were categorized according to the Compendium and the associated measured metabolic equivalent (MET) values were used to calculate EEE for all activity listed (2024 adult compendium). It is important to note that Polar watch exercise data was ultimately not included in this analysis due to technical errors in uploading data beyond the control of study staff.

Energy Availability

Energy availability (EA) was calculated according to the equations EA=(EI-EEE)/kg fat-free mass (FFM) in kcal/kg and EA=(EI-EEE)/kg lean body mass (LBM) in kcal/kg. EI was determined by information entered by participants into the MyFitnessPal application during the 3-day diet log entry period. EEE was determined by kilocalorie expenditure calculated by the Polar watch during the 7-day exercise log and activity recording period; specifically, data from the same 3 days as the diet log entry was used for the purposes of calculating energy availability. FFM and LBM were determined by the DXA scan for body composition and used to normalize the difference in EI and EEE.

Aerobic Fitness (VO2max)

To assess aerobic fitness, a VO2max test was administered during each training phase, indicating participants' physical condition and performance during each phase of their competitive season. Using a modified Astrand protocol, participants first chose a pace to run at on a treadmill (Fullvision Trackmaster, Newton, KS, USA), approximating a pace that would exhaust them on a 20-minute run. After a 2–5-minute warmup, participants began running at the selected pace at a 0% incline; for the first 6 minutes, the incline was increased by study staff by 2% every 2 minutes, and then by 1% every 1 minute until the participant reached maximum exertion and the test was complete. Participants then walked on the treadmill for at least 5 minutes to cool down.

Oxygen uptake was measured using indirect calorimetry via a mouthpiece secured with no air leakage through the nose and a metabolic cart (Cosmed Quark RMR, Rome, Italy)

measuring VO2 consumed under maximum exertion while running on the treadmill. Rate of perceived exertion (RPE) (Borg, 1970), on a scale of 6 (very, very light) to 20 (very, very hard), was measured and recorded by study staff by holding up a scale for the participant to point to before moving on to each % incline increase. A Polar heart rate monitor, consisting of a strap around the chest and a corresponding watch displaying the heart rate, was used for study staff to monitor the participant's heart rate throughout the test. Blood pressure was also taken before and after the test to ensure return to baseline.

Resting Metabolic Rate

For the resting metabolic rate (RMR) assessment visit, participants arrived at the lab between 6:00-8:30 AM in the fasted state (12-hour overnight fast), having refrained from heavy exercise, caffeine, alcohol, tobacco, and herbal medications for 24 hours. First, body weight and height were measured. The subject was asked to lie supine at rest for 30-45 minutes to achieve a steady resting state before the RMR test. RMR was then measured for 30-45 minutes via indirect calorimetry utilizing a Cosmed Quark RMR metabolic cart. Oxygen consumption (VO2) and carbon dioxide production (VCO2) were assessed every 30 seconds during the test for determination of RMR. The Weir equation (Weir, 1949) was then used to calculate RMR using data for VO2 and CO2.

Energy status was determined using a DXA-derived RMR ratio, or the ratio of measured RMR to predicted RMR using the equation previously described (Strock et al., 2020). RMR ratio serves as a biomarker of energy deficiency; a low ratio represents metabolic compensation via a suppressed metabolic rate (Strock et al., 2020). This ratio has been found to successfully predict

low triiodothyronine (TT3), the active thyroid hormone that plays a large role in metabolism, in a study of exercising women (Strock et al., 2020). RMR (kcal/kg LBM/day) was also reported to reflect the reduction of RMR per kilogram of tissue below a certain ratio threshold. Those with a DXA-derived RMR ratio of <0.94 were defined as energy deplete, and those ≥0.94 were defined as energy replete, based on a previous study (Strock et al., 2020).

Assessment of Stress

Psychological Stress

Participants' stress was assessed as part of the study. The Perceived Stress Scale (PSS) (Cohen et al., 1983) and Recovery-Stress Questionnaire for Athletes (RESTQ-52) (Kellmann and Kallus, 2001) were administered via REDCap and assessed stressful events and perceived psychological stress, and life and sport-related stress, respectively. These scales were compared across different phases of a competitive season (pre-, peak-, and off-) to further assess changes in activity level and stress.

The PSS is a 14-item questionnaire measuring the degree of stress appraised by individuals in various situations and has been validated against other measures of stress such as life-event scores, depressive and physical symptoms, and social anxiety (Cohen et al., 1983). This scale has shown adequate reliability to measure experienced levels of stress and the role of appraised stress in disease and behavior etiology per several studies in college students and smoking-cessation program participants (Cohen et al., 1983).

The RESTQ-52 is a shortened version of the RESTQ-76 Sport, designed after the original 48-item Recovery-Stress Questionnaire to measure the frequency of current stress and the frequency of activities associated with recovery, assessing consequences of stressful and restful events during the past three days/nights (Kellmann and Kallus, 2001). It consists of several subscales measuring general stress, general recovery, sport-related stress, and sport recovery to generate a final score indicating balance of stress vs recovery (Kellmann and Kallus, 2001). The RESTQ-52 has been found to have high test-retest reliability over a 24-hour period, and both construct and criterion validity; relationships between actual state and this questionnaire have been empirically verified (Kellmann and Kallus, 2001).

Hair Cortisol

Hair cortisol was assessed as a chronic measurement of cortisol in the body, associated with stress. For this procedure, a sample of hair was cut from the back of the head, as close to the scalp as possible, approximately 2 cm below the cranial bone. Samples taken were about 4 follicles in height and 1 inch wide at the scalp, with the overall diameter of the hair bundle similar to half the diameter of a pencil. The strands were placed onto aluminum foil with the scalp end clearly marked, and aluminum foil was folded over sample and secured with a paper clip. Samples were stored in a dry and dark location before being shipped to the Dresden Lab in Germany, under direction of Dr. Clemens Kirschbaum, for processing.

For processing, hair samples were cut 3 cm from the scalp end and analyzed as a cumulative measure of cortisol levels over the past 3 months, based on an average hair growth rate of 1cm/month (Gao et al., 2013). Hair samples were washed in isopropanol at room

temperature and dried under a fume hood for at least 12 h (Gao et al., 2013). Whole, non-pulverized hair was weighed, internal standard and methanol were added, and hair was incubated for 18 h at room temperature to extract steroids (Gao et al., 2013). Samples were then spun in a centrifuge, dried under a constant stream of nitrogen, and resuspended in distilled water (Gao et al., 2013).

Finally, samples were analyzed for cortisol and cortisone content, measured in pg/mg with a lower detection limit of 0.3 pg/mg, in a single batch using a HPLC-MS/MS system consisting of a Shimadzu LC-20AD HPLC unit, a Shimadzu SIL-20AC autosampler and a Shimadzu CTO-20AC column temperature oven (Shimadzu, Canby, OR, USA) (Gao et al., 2013). The mass spectrometer used was an AB Sciex API 5000 Turbo-ion-spray triple quadrupole tandem mass spectrometer with atmospheric pressure chemical ionization (APCI) ion source (AB Sciex, Foster City, CA, USA), controlled by AB Sciex Analyst software (version 1.5.1). To clean samples prior to injection of analytes to the analytical column, a Chromolith Speed ROD RP-18e HPLC column from Merck KGaA (Darmstadt, Germany) was used. The analytical column used was a Shim-pack XR-ODS LC column from Shimadzu with a Phenomenex security guard column (Aschaffenburg, Germany) (Gao et al., 2013). Variables of interest for analysis included: hair cortisol concentration (pg/mL), hair cortisone concentration (pg/mL), and the ratio of cortisol:cortisone concentrations. Cortisone is the inactive metabolite of cortisol, so the hair cortisol/cortisone ratio represents stress exposure by indicating how the body manages stress, whereby higher ratio values indicate a lower conversion of cortisol to cortisone and potentially a higher stress load on the body (Zhang et al., 2017; Dötsch et al., 2001).

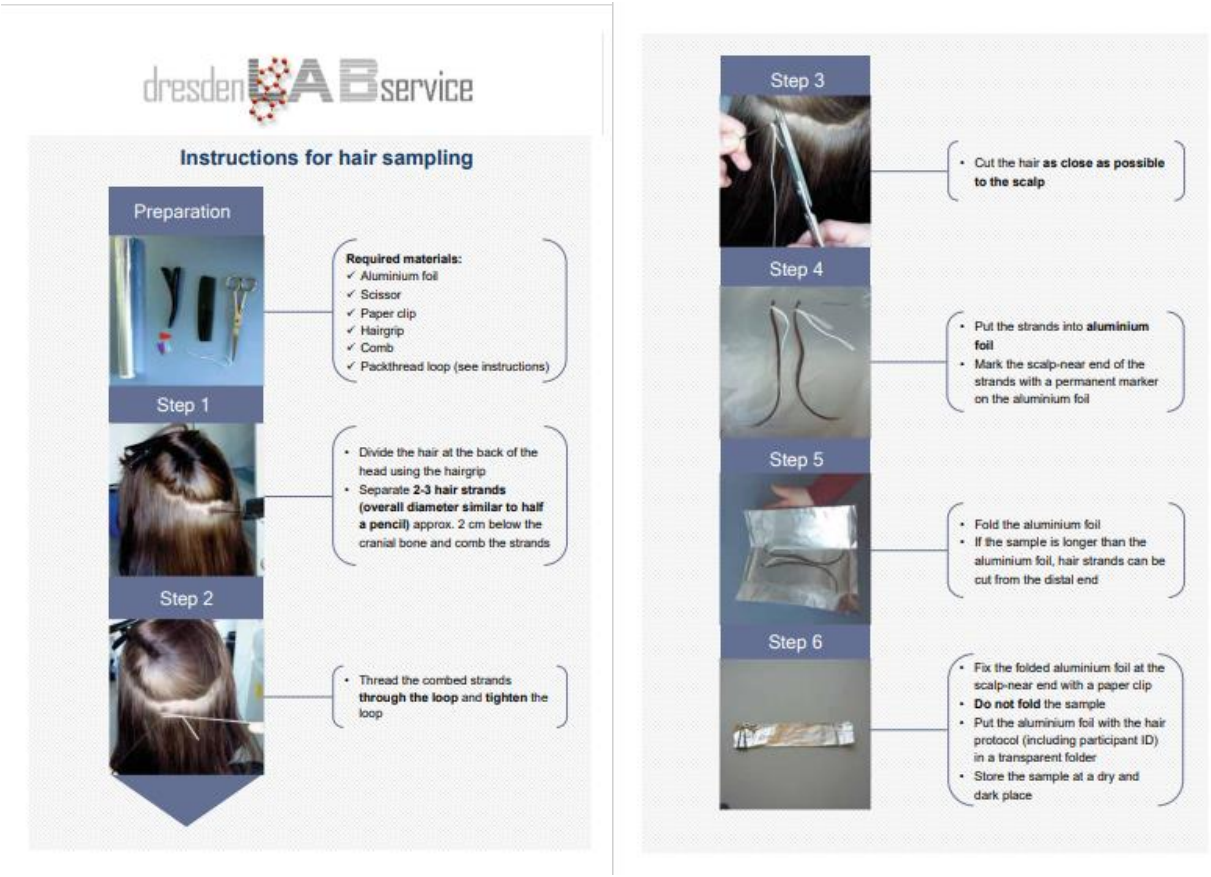

Figure 2. Instructions for hair sampling for cortisol assessment, provided by Dresden Laboratory under the direction of Dr. Clemens Kirschbaum.

Statistical Analysis

The data was analyzed using SPSS Statistics Software (version 29 Chicago, IL). Before analysis, data were screened for outliers and each variable was verified for normality and variance. Normality was tested using the Shapiro-Wilk statistic. Independent t-tests were used for comparisons of demographic and anthropometric data, as well as variables of interest within each training phase to determine statistically significant differences between sexes at each phase of the competitive season.

In order to test the hypothesis that hair cortisol reflects metabolic, physical, and psychological stress across a competitive season, a generalized linear mixed effects model (GLMM) was run. This model used subject ID as the subject, hair cortisol as the target (dependent variable), and each stress variable as a fixed effect along with time (coded for pre-, peak-, and off-season). Each stress variable was run independently of each other in order to evaluate and compare effects on hair cortisol and avoid covariance among closely related variables such as RMR/kg FFM and RMR ratio.

Spearman's rho correlations were performed to evaluate the relationships between stress variables, including metabolic (RMR/kg FFM and DXA RMR ratio), physical (training volume in exercise mins/wk and EEE), and psychological stress (PSS score and RESTQ stress subscales), and hair cortisol and hair cortisol/cortisone ratio. Because hair cortisol and cortisol/cortisone ratio were not normally distributed for all three timepoints, Spearman's rho was chosen over Pearson correlations in order to evaluate them non-parametrically.

Chapter 3

Results

Athlete Characteristics

The athletes in this study consisted of 19 NCAA Division I or university-affiliated club sport athletes (10 females and 9 males). Ten sports were represented, including: cross country, swimming, triathlon, rugby, lacrosse, gymnastics, cheerleading, dance, diving, and powerlifting. Athletes were further categorized by sport-type, as either: leanness/aerobic sports (including team sports) (cross country, swimming, triathlon, rugby, and lacrosse; n=11 total, 7 males and 4 females), or aesthetic/weight class sports (gymnastics, cheerleading, dance, diving, and powerlifting; n=8 total, 2 males and 6 females). There were no dropouts. Of the 19 athletes included in this study, 16 athletes identified themselves as Caucasian and 3 athletes identified themselves as Asian. Further descriptive information is included in Table 1.

Descriptive Demographic, Exercise, Energy, Stress, and Hair Metabolite Characteristics

Pre-Season

Average demographic variables and anthropometric measures for athletes during pre-season are presented in Table 1. Male athletes had significantly greater height, fat-free mass, and lean body mass compared to female athletes. Female athletes had significantly greater total body fat percentage and fat mass compared to male athletes.

Average exercise session frequency, training volume, duration, and exercise energy expenditure (EEE) during pre-season, determined by 7-day exercise log and compendium, are included in Table 1. During pre-season, athletes trained an average of 6 days/wk and 8 sessions/wk, and had an average exercise energy expenditure of 590 kcal/d. There were no significant differences between male and female athletes for any of these measures.

Average energy and metabolism measures for all athletes during pre-season are depicted in Table 1. There were no significant differences between male and female athletes for either energy intake or energy availability measures. Not surprisingly, male athletes had significantly higher measured RMR during pre-season compared to female athletes (1531.18 ± 204.44 vs. 1265.23 ± 198.49, p=0.02).

Athletes' stress measures for pre-season, including both psychological stress questionnaire scores and physiological stress (from hair samples) are outlined in Table 1. Female athletes had a significantly higher drive for leanness than male athletes (42 ± 2 vs. 37 ± 3, p=0.001). All other psychological stress measures demonstrated no significant differences between males and females during the pre-season, although higher RESTQ general stress and total stress in females vs. males were nearly significant (p=0.058 for both). Cortisol, cortisone, and cortisol/cortisone ratio measurements were not significantly different between male and female athletes in the pre-season.

Peak-Season

Average demographic variables and anthropometric measures for athletes during peak-season are presented in Table 2. Male athletes had significantly greater height, fat-free mass, and

lean body mass compared to female athletes. Female athletes had significantly greater total body fat percentage and fat mass compared to male athletes.

Average exercise session frequency, training volume, duration, and exercise energy expenditure (EEE) during peak-season, determined by 7-day exercise log and compendium, are included in Table 2. During peak-season, athletes trained an average of 6 days/wk and 8 sessions/wk, and had an average exercise energy expenditure of 671 kcal/d. There were no significant differences between male and female athletes for any of these measures. Male athletes had significantly higher total daily energy expenditure (TDEE) during peak-season than female athletes (2799.87 ± 783.21 vs. 2106.91 ± 497.67, p=0.036).

Average energy and metabolism measures for all athletes during peak-season are depicted in Table 2. There were no significant differences between male and female athletes for either energy intake or energy availability measures. Male athletes had significantly higher measured RMR during peak-season compared to female athletes (1805.45 ± 392.43 vs. 1365.38 ± 183.02, p=0.006).

Athletes' stress measures for peak-season, including both psychological stress questionnaire scores and physiological stress (from hair samples) are outlined in Table 2. Males had a significantly higher average RESTQ sport-specific recovery compared to female athletes (14 ± 4 vs. 11 ± 3, p=0.017). Additionally, females generally had higher RESTQ general stress than males (p=0.051), and males generally had higher RESTQ total recovery and final scores than females (p=0.052 and p=0.055, respectively), although these differences were not quite

significant. For all other stress measures, there were no significant differences between males and females during the peak-season training phase.

Off-Season

Average demographic variables and anthropometric measures for athletes during off-season are presented in Table 3. Male athletes had significantly greater height, fat-free mass, and lean body mass compared to female athletes. Female athletes had significantly greater total body fat percentage and fat mass compared to the male athletes.

Average exercise session frequency, training volume, duration, and exercise energy expenditure (EEE) during off-season, determined by 7-day exercise log and compendium, are included in Table 3. During off-season, athletes trained an average of 5 days/wk and 10 sessions/wk, and expended an average of 595 kcal/d due to exercise. There were no significant differences between male and female athletes for any of these measures, including TDEE.

Average energy and metabolism measures for all athletes during off-season are depicted in Table 3. There were no significant differences between male and female athletes for either energy intake or energy availability measures, nor measures of metabolism.

Athletes' stress measures for off-season, including both psychological stress questionnaire scores and physiological stress (from hair samples) are outlined in Table 3. Female athletes had a significantly higher drive for leanness than male athletes (43 ± 6 vs. 37 ± 6,

p=0.05). All other stress measures exhibited no significant differences between males and females during the off-season phase.

Table 1 Pre-season descriptive data.

	All (n=19)				Male (n=9)				Female (n=10)				
	Mean	±	SD	Range	Mean	±	SD	Range	Mean	±	SD	Range	P value
Demographics and Anthropometrics													
Age (yr)	21	±	2	18-27	21	±	3	18-27	20	±	1	19-22	0.371
Height (cm)	169.61	±	6.07	158.00-176.70	173.72	±	2.38	171.00-176.70	165.91	±	6.05	158.00-175.00	**0.003**
Weight (kg)	67.68	±	9.07	53.65-88.00	69.93	±	10.43	58.75-88.00	65.68	±	7.73	53.65-76.35	0.351
BMI (kg/m^2)	23.50	±	2.85	18.80-28.20	23.19	±	3.42	18.80-28.20	23.80	±	2.42	21.10-26.70	0.684
Total Body Fat (%)	24.41	±	6.45	16.30-36.10	18.89	±	2.41	16.30-23.60	29.32	±	4.51	24.80-36.10	**<0.001**
Fat Mass (kg)	16.28	±	4.61	9.96-23.66	13.13	±	3.15	9.96-18.36	19.08	±	3.89	13.70-23.66	**0.004**
Fat Free Mass (kg)	50.54	±	8.27	38.46-68.97	55.79	±	7.41	46.53-68.97	45.87	±	6.06	38.46-56.82	**0.008**
Lean Body Mass (kg)	47.82	±	7.95	36.05-65.12	52.94	±	6.96	44.22-65.12	43.27	±	5.86	36.05-53.65	**0.007**
Exercise Training													
Session Frequency (sessions/d)	8	±	4	4-18	9	±	5	4-18	7	±	3	5-16	0.472
Training Volume (mins/wk)	606	±	329	189-1295	572	±	271	189-1010	636	±	388	210-1295	0.703
Average Duration (mins/d)	117	±	59	47-283	113	±	74	47-283	120	±	47	55-185	0.819
Exercise Frequency (d/wk)	6	±	1	3-7	6	±	1	4-7	5	±	2	3-7	0.658
Average EEE (compendium) (kcal/d)	590	±	273	265-1109	596	±	224	339-999	585	±	319	265-1109	0.939
Energy Intake													
Energy Intake (avg kcal/d)	2321.20	±	543.19	1307.53-3090.17	2525.43	±	425.02	1903.37-3073.97	2139.65	±	594.36	1307.53-3090.17	0.149
Energy Availability (kcal/kg FFM/d)	39.24	±	13.23	20.27-68.60	39.11	±	10.22	21.32-51.56	39.35	±	16.09	20.27-68.60	0.971
Metabolism													
Measured RMR (kcal/d)	1381.58	±	237.27	960.55-1784.00	1531.18	±	204.44	1247.14-1784.00	1265.23	±	198.49	960.55-1550.02	**0.02**
RMR per kg Fat Free Mass (kcal/kg FFM/d)	27.78	±	3.41	22.99-34.98	27.93	±	3.73	23.69-34.98	27.66	±	3.36	22.99-31.87	0.884
DXA-Derived RMR Ratio	0.93	±	0.10	0.80-1.13	0.92	±	0.10	0.81-1.13	0.94	±	0.10	0.80-1.07	0.754
TDEE (kcal/d)	1962.59	±	321.89	1397.70-2405.78	2124.51	±	234.61	1693.95-2405.78	1820.91	±	333.00	1397.70-2245.73	0.066
Stress Questionnaires													
Perceived Stress (PSS)	32	±	4	22-38	31	±	5	22-38	33	±	3	27-37	0.308
General Stress (RESTQ)	11	±	5	4-21	8	±	3	4-14	13	±	5	7-21	*0.058*
General Recovery (RESTQ)	18	±	4	11-25	19	±	4	11-25	17	±	3	13-22	0.424
Sport-specific Stress (RESTQ)	4	±	2	1-9	4	±	2	1-6	5	±	2	1-9	0.123
Sport-specific Recovery (RESTQ)	13	±	4	8-22	14	±	3	8-18	13	±	5	8-22	0.7
Total Stress (RESTQ)	15	±	7	5-28	12	±	4	5-17	18	±	7	8-28	*0.058*
Total Recovery (RESTQ)	31	±	8	18-43	32	±	7	18-43	30	±	8	21-43	0.542
Final Score (RESTQ)	16	±	12	- 3-35	21	±	9	1-29	12	±	13	-3-35	0.147
Drive for Leanness	40	±	4	34-45	37	±	3	34-43	42	±	2	40-45	**0.001**
Drive for Muscularity	45	±	8	32-57	43	±	8	34-57	47	±	8	32-56	0.306
Hair Metabolites													
Cortisol (pg/mg)	4.98	±	2.55	1.58-12.27	3.99	±	1.83	1.58-6.34	5.96	±	2.91	3.33-12.27	0.155
Cortisone (pg/mg)	12.67	±	4.87	4.99-23.38	12.19	±	3.93	7.42-18.23	13.16	±	5.95	4.99-23.38	0.725
Cortisol/Cortisone Ratio	0.42	±	0.21	0.21-0.83	0.33	±	0.16	0.21-0.65	0.51	±	0.23	0.22-0.83	0.719

Table 2 Peak-Season descriptive data.

	All (n=19)				Male (n=9)				Female (n=10)				
	Mean	±	SD	Range	Mean	±	SD	Range	Mean	±	SD	Range	P value
Demographics and Anthropometrics													
Age (yr)	21	±	2	18-27	21	±	3	18-27	20	±	1	19-22	0.371
Height (cm)	169.61	±	6.07	158.00-176.70	173.722	±	2.379	171.00-176.70	165.91	±	6.0455	158.00-175.00	**0.003**
Weight (kg)	67.81	±	8.99	55.30-88.00	69.9611	±	10.14996	58.00-88.00	65.865	±	7.83667	55.30-77.20	0.336
BMI (kg/m^2)	23.59	±	2.90	18.60-28.20	23.2	±	3.3612	18.60-28.20	23.94	±	2.55	21.30-28.10	0.593
Total Body Fat (%)	24.26	±	6.26	16.90-35.60	18.822	±	2.3853	16.90-23.70	29.16	±	4.1404	24.60-35.60	**<0.001**
Fat Mass (kg)	15.89	±	4.39	10.15-23.44	12.935711	±	3.091407	10.15-19.15	18.54696	±	3.6767917	13.35-23.44	**0.002**
Fat Free Mass (kg)	49.81	±	8.27	38.56-69.28	55.307656	±	7.4379495	46.24-69.28	44.86618	±	5.5119428	38.56-55.45	**0.003**
Lean Body Mass (kg)	47.12	±	7.92	36.15-65.39	52.452522	±	6.9986644	43.98-65.39	42.32063	±	5.2836088	36.15-52.29	**0.002**
Exercise Training													
Session Frequency (sessions/d)	8	±	3	3-14	9	±	3	6-14	8	±	4	3-13	0.409
Training Volume (mins/wk)	671	±	316	93-1380	632	±	371	250-1380	686	±	298	93-1093	0.834
Average Duration (mins/d)	133	±	46	57-219	120	±	51	57-197	146	±	39	81-219	0.227
Exercise Frequency (d/wk)	6	±	1	3-7	6	±	1	3-7	6	±	1	4-7	0.728
Average EEE (compendium) (kcal/d)	603	±	247	294-1187	605	±	270	294-1187	602	±	240	302-1090	0.98
Energy Intake													
Energy Intake (avg kcal/d)	2324.98	±	698.72	945.00-3508.07	2591.61	±	691.37	1517.00-3508.07	2085.01	±	645.34	945.00-3115.00	0.117
Energy Availability (kcal/kg FFM/d)	40.87	±	10.87	18.93-58.83	41.57	±	9.19	29.42-58.83	40.24	±	12.67	18.93-55.47	0.798
Metabolism													
Measured RMR (kcal/d)	1560.96	±	363.01	1126.51-2582.00	1805.45	±	392.43	1483.00-2582.00	1365.38	±	183.02	1126.51-1720.11	**0.006**
RMR per kg Fat Free Mass (kcal/kg FFM/d)	31.80	±	5.19	25.44-45.08	33.19	±	5.84	27.31-45.08	30.69	±	4.60	25.44-38.46	0.323
DXA-Derived RMR Ratio	1.04	±	0.16	0.85-1.46	1.10	±	0.19	0.87-1.46	0.99	±	0.11	0.85-1.21	0.141
TDEE (kcal/d)*	2414.89	±	713.61	1368.14-4342.00	2799.87	±	783.21	1973.43-4342.00	2106.91	±	497.67	1368.14-2746.36	**0.036**
Stress Questionnaires													
Perceived Stress (PSS)	34	±	4	28-40	34	±	4	29-39	33	±	4	28-40	0.608
General Stress (RESTQ)	14	±	7	7-28	11	±	5	7-23	17	±	8	7-28	*0.051*
General Recovery (RESTQ)	16	±	5	8-26	18	±	5	8-26	15	±	4	10-22	0.145
Sport-specific Stress (RESTQ)	6	±	4	2-13	5	±	3	2-13	7	±	4	2-13	0.368
Sport-specific Recovery (RESTQ)	12	±	4	7-21	14	±	4	9-21	11	±	3	7-15	**0.017**
Total Stress (RESTQ)	20	±	11	9-41	16	±	8	9-36	24	±	11	11-41	0.092
Total Recovery (RESTQ)	29	±	8	17-46	33	±	9	17-46	25	±	6	19-35	*0.052*
Final Score (RESTQ)	9	±	18	-22-35	17	±	16	-19-35	2	±	17	-22-24	*0.055*
Drive for Leanness	41	±	7	29-54	39	±	8	30-54	42	±	6	29-51	0.331
Drive for Muscularity	45	±	9	28-67	43	±	10	28-59	47	±	8	39-67	0.376
Hair Metabolites													
Cortisol (pg/mg)	6.43	±	5.83	0.50-16.78	7.83	±	7.06	0.88-16.78	5.35	±	4.83	0.50-14.83	0.417
Cortisone (pg/mg)	11.88	±	5.44	2.61-18.85	11.90	±	5.29	5.94-17.54	11.87	±	5.88	2.61-18.85	0.99
Cortisol/Cortisone Ratio	0.39	±	0.25	0.13-1.01	0.37	±	0.29	0.13-1.01	0.40	±	0.23	0.18-0.78	0.494

Table 3 Off-season descriptive data.

	All (n=19)				Male (n=9)				Female (n=10)				
	Mean	±	SD	Range	Mean	±	SD	Range	Mean	±	SD	Range	P value
Demographics and Anthropometrics													
Age (yr)	21	±	2	18-27	21	±	3	18-27	20	±	1	19-22	0.371
Height (cm)	169.61	±	6.07	158.00-176.70	173.72	±	2.38	171.00-176.70	165.91	±	6.05	158.00-175.00	**0.003**
Weight (kg)	67.60	±	9.01	53.60-86.06	70.63	±	10.15	58.50-86.06	65.51	±	8.45	53.60-77.20	0.326
BMI (kg/m^2)	23.58	±	2.95	18.70-28.40	23.33	±	3.31	18.70-27.60	24.00	±	2.90	21.40-28.40	0.548
Total Body Fat (%)	25.39	±	6.37	17.50-36.80	20.01	±	3.56	17.50-27.60	30.09	±	4.34	24.80-36.80	**<0.001**
Fat Mass (kg)	16.84	±	4.80	10.54-25.84	14.05	±	4.40	10.54-22.14	19.42	±	3.93	14.16-25.84	**0.013**
Fat Free Mass (kg)	49.48	±	7.72	38.99-67.93	55.10	±	6.74	46.88-67.93	45.00	±	5.90	38.99-56.82	**0.006**
Lean Body Mass (kg)	46.81	±	7.37	36.98-64.12	52.27	±	6.23	44.65-64.12	42.47	±	5.66	36.98-53.65	**0.005**
Exercise Training													
Session Frequency (sessions/d)	10	±	5	2-18	12	±	5	5-18	8	±	4	2-15	0.138
Training Volume (mins/wk)	595	±	331	144-1260	536	±	231	178-960	647	±	407	144-1260	0.496
Average Duration (mins/d)	100	±	40	45-180	84	±	27	45-137	114	±	45	51-180	0.114
Exercise Frequency (d/wk)	5	±	2	1-7	6	±	1	4-7	4	±	2	1-7	0.076
Average EEE (compendium) (kcal/d)	522	±	265	191-1009	480	±	164	252-749	558	±	339	191-1009	0.576
Energy Intake													
Energy Intake (avg kcal/d)	2385.16	±	649.94	1471.83-3754.27	2670.04	±	625.40	1471.83-3754.27	2131.94	±	591.04	1502.73-2859.27	0.088
Energy Availability (kcal/kg FFM/d)	43.90	±	11.47	21.67-61.77	45.15	±	11.55	21.67-61.78	42.79	±	11.98	28.53-58.71	0.685
Metabolism													
Measured RMR (kcal/d)	1739.89	±	341.75	1336.14-2576.00	1882.49	±	369.38	1568.16-2576.00	1597.29	±	259.52	1336.14-2077.10	0.096
RMR per kg Fat Free Mass (kcal/kg FFM/d)	35.81	±	6.44	26.55-52.32	34.48	±	4.75	28.24-42.74	37.14	±	7.90	26.55-52.32	0.428
DXA-Derived RMR Ratio	1.19	±	0.22	0.88-1.74	1.15	±	0.18	0.93-1.49	1.23	±	0.26	0.88-1.74	0.518
TDEE (kcal/d)**	2235.55	±	603.85	1357.77-3858.00	2382.61	±	651.08	1959.37-3858.00	2088.48	±	555.00	1357.77-3177.95	0.347
Stress Questionnaires													
Perceived Stress (PSS)	32	±	5	19-39	31	±	6	19-39	32	±	4	27-38	0.644
General Stress (RESTQ)	11	±	6	4-28	10	±	3	4-13	12	±	7	4-28	0.31
General Recovery (RESTQ)	19	±	4	12-26	19	±	4	14-26	18	±	4	12-22	0.499
Sport-specific Stress (RESTQ)	4	±	2	0-9	4	±	2	1-7	5	±	3	0-9	0.76
Sport-specific Recovery (RESTQ)	11	±	4	6-21	11	±	5	7-21	11	±	4	6-18	0.763
Total Stress (RESTQ)	15	±	7	4-37	14	±	4	7-20	17	±	9	4-37	0.394
Total Recovery (RESTQ)	30	±	7	19-47	30	±	8	21-47	30	±	6	19-39	0.59
Final Score (RESTQ)	15	±	12	-9-41	17	±	11	6-41	12	±	13	-9-24	0.385
Drive for Leanness	40	±	7	28-49	37	±	6	31-49	43	±	6	30-49	**0.05**
Drive for Muscularity	43	±	12	26-78	46	±	15	32-78	41	±	9	26-58	0.577
Hair Metabolites													
Cortisol (pg/mg)	6.51	±	6.28	1.45-25.36	3.81	±	2.25	1.45-7.33	9.20	±	7.93	1.94-25.36	0.101
Cortisone (pg/mg)	14.68	±	8.02	5.69-31.42	12.43	±	4.56	7.53-20.94	16.92	±	10.27	5.69-31.42	0.285
Cortisol/Cortisone Ratio	0.40	±	0.23	0.18-1.07	0.29	±	0.09	0.18-0.47	0.51	±	0.28	0.21-1.07	0.619

Metabolic, physical, and psychological stress as predictors of hair cortisol across the competitive season

A generalized linear mixed effects model (GLMM) was run to test the hypothesis that hair cortisol reflects metabolic, physical, and psychological stress across a competitive season. Indicators of stress (RMR/kg FFM and RMR ratio indicating metabolic stress, exercise mins/wk and EEE indicating physical stress, and PSS score and RESTQ stress subscales indicating psychological stress) were run as fixed effects along with time (indicating pre-, peak-, and off-seasons), and the relationships between each of these variables and hair cortisol was analyzed. None of the outputs from this model demonstrated statistical significance between any of the stressors and hair cortisol across time.

Relationships between metabolic, physical, and psychological stress and hair cortisol within each season phase

In addition to the GLMM model, we ran correlations between hair cortisol and each metabolic, physical, and psychological stress variable to determine relationships between variables. These correlations were separated out by timepoint; i.e. pre-season stress variables were correlated with pre-season hair cortisol. In addition to hair cortisol, we analyzed relationships between each stress variable and hair cortisol/cortisone ratio, another marker of stress load.

It is important to note that analyses of the relationships between hair cortisol (and cortisol/cortisone ratio) and metabolic, physical, and psychological stress were determined under the assumption that a 3 cm hair sample captures cortisol exposure in the previous 3 months to the time of each hair sample collection. Due to the heterogenous nature of the sports represented in this study, time between each season phase varied considerably, presenting a challenge when determining if hair cortisol taken at each season phase would reflect stress exposure within that season or during the previous season. Because the average days in between each seasonal time point exceeded 3 months (90 days) (Table 4), we decided to analyze these relationships according to the assumption that hair cortisol would reflect stress exposure within the same season phase. However, future studies would benefit from more complex analyses of the impact of time-between-seasons variation on the interpretation of results.

Note that 3 of the athletes participating in this study completed their pre-season timepoint measurements after the off-season timepoint rather than before peak-season due to the timing of their specific sport season with when they joined the study. This is included in Table 4 below.

Table 4 Average time between hair cortisol samples in each season phase (days).

Pre-Peak (days)					Peak-Off (days)					Off-Pre (days)				
Mean	±	SD	Range	N	Mean	±	SD	Range	N	Mean	±	SD	Range	N
145	±	69	40-263	14	124	±	59	31-211	14	137	±	80	53-211	3

Pre-Season

Correlations between metabolic, physical, and stress variables and hair cortisol, as well as hair cortisol/cortisone ratio, within the pre-season for all athletes are included in Table 5 below. There were no statistically significant relationships ($p<0.05$) between any of these stress variables and hair cortisol or cortisol/cortisone ratio.

Table 5 Correlations between hair cortisol and cortisol/cortisone ratio and metabolic, physical, and psychological stress variables during the pre-season.

Pre-Season		Hair Cortisol (pg/mg)			Hair Cortisol/Cortisone Ratio		
		N	r	p	N	r	p
Metabolic stress	RMR/kg FFM (kcal/kg/d)	13	0.088	0.775	13	0.258	0.394
	DXA RMR Ratio	13	0.055	0.859	13	0.176	0.566
Physical stress	Training volume (mins/wk)	14	-0.169	0.563	14	-0.007	0.982
	EEE (kcal/day)	13	0.088	0.775	13	0.412	0.162
Psychological stress	PSS Score	14	0.066	0.822	14	-0.033	0.91
	RESTQ General Stress	14	-0.051	0.863	14	-0.219	0.453
	RESTQ Sport Stress	14	-0.024	0.935	14	-0.125	0.67
	RESTQ Total Stress	14	-0.02	0.946	14	-0.191	0.513

Peak-Season

Correlations between metabolic, physical, and stress variables and hair cortisol, as well as hair cortisol/cortisone ratio, within the peak-season for all athletes are included in Table 6 below. There were no statistically significant relationships ($p<0.05$) between any of these stress variables and hair cortisol or cortisol/cortisone ratio.

Table 6 Correlations between hair cortisol and cortisol/cortisone ratio and metabolic, physical, and psychological stress variables during the peak-season.

Peak-Season		Hair Cortisol (pg/mg)			Hair Cortisol/Cortisone Ratio		
		N	r	p	N	r	p
Metabolic stress	RMR/kg FFM (kcal/kg/d)	16	0.082	0.762	16	0.396	0.129
	DXA RMR Ratio	16	0.112	0.68	16	0.429	0.098
Physical stress	Training volume (mins/wk)	16	-0.432	0.094	16	0.115	0.672
	EEE (kcal/day)	16	-0.426	0.099	16	-0.053	0.845
Psychological stress	PSS Score	16	0.001	0.996	16	0.202	0.453
	RESTQ General Stress	14	0.482	0.081	16	-0.151	0.577
	RESTQ Sport Stress	14	0.275	0.341	16	0.321	0.226
	RESTQ Total Stress	14	0.459	0.098	16	0.071	0.794

Off-Season

Correlations between metabolic, physical, and stress variables and hair cortisol, as well as hair cortisol/cortisone ratio, within the off-season for all athletes are included in Table 7 below. Both RMR/kg FFM and DXA-derived RMR ratio had significant positive relationships with hair cortisol ($r=0.6$, $p=0.018$ and $r=0.646$, $p=0.009$ respectively). In addition, Both RMR/kg FFM and DXA-derived RMR ratio had significant positive relationships with hair cortisol/cortisone ratio ($r=0.643$, $p=0.01$ and $r=0.564$, $p=0.028$ respectively). No physical or psychological stress variables had a statistically significant relationship with either hair cortisol or cortisol/cortisone ratio.

Table 7 Correlations between hair cortisol and cortisol/cortisone ratio and metabolic, physical, and psychological stress variables during the off-season.

Off-Season		Hair Cortisol (pg/mg)			Hair Cortisol/Cortisone Ratio		
		N	r	p	N	r	p
Metabolic stress	RMR/kg FFM (kcal/kg/d)	15	0.6	**0.018**	15	0.643	**0.01**
	DXA RMR Ratio	15	0.646	**0.009**	15	0.564	**0.028**
Physical stress	Training volume (mins/wk)	16	-0.224	0.405	16	-0.232	0.387
	EEE (kcal/day)	14	-0.2	0.493	14	-0.037	0.899
Psychological stress	PSS Score	16	0.162	0.548	16	0.024	0.931
	RESTQ General Stress	16	0.05	0.854	16	-0.084	0.757
	RESTQ Sport Stress	16	-0.041	0.879	16	-0.139	0.608
	RESTQ Total Stress	16	0.047	0.863	16	-0.065	0.812

Chapter 4

Discussion

The literature is currently limited in terms of studies evaluating stress changes across a competitive season. This longitudinal observational study is unique in that it took advantage of following individuals over time and comparing measures of stress from pre- to peak- to off-season for the same cohort of athletes, allowing for a meaningful interpretation of how stress changes over time. This study took into consideration the notion that participating in collegiate sports presents a multifactorial stress environment and aimed to evaluate the effects of multiple sources of stress, namely metabolic, physical, and psychological, on overall cortisol exposure. By investigating several such variables likely to contribute to higher overall stress and therefore, higher circulating cortisol levels, we were able to evaluate the use of hair cortisol as a biological marker of this stress exposure over time in athletes.

Our initial model testing the hypothesis that hair cortisol reflects metabolic, physical, and psychological stress across a competitive season did not yield any significant relationships between individual stress variables falling into these categories and hair cortisol over time as we would have expected. So, we ran correlations between individual stress variables and hair cortisol, as well as cortisol/cortisone ratio, within each season phase to identify more specific relationships. Surprisingly, we did not find many significant relationships; the only ones found were between metabolic stress variables and hair cortisol and cortisol/cortisone ratio in the off season, but no other seasons. Further, we would have expected indicators of metabolic stress due to energy deficiency, namely low RMR/kg FFM and RMR ratio, to correlate with higher hair cortisol due to cortisol's role as a counterregulatory hormone that is often upregulated when

energy stores are low (Thavaraputta et al., 2023). However, we found a positive relationship between metabolic variables and hair cortisol in the off season, indicating that a higher RMR and RMR ratio correlate with more stress and higher circulating cortisol levels, opposite of expected. A possible explanation for this could be that increased training volume during the peak-season phase, in combination with an energy surplus as indicated by RMR ratio >0.94 (Strock et al., 2020), drove up RMR which continued into the off-season. Therefore, RMR in this sense could have demonstrated more physical stress due to effects of exercise on increasing RMR (Molé, 1990), therefore increasing cortisol.

Overall, we did not find that hair cortisol reflected physical or psychological stress across a competitive season and found a limited use for hair cortisol as a reflection of metabolic stress. However, addressing limitations of this study in future studies could have dramatic impacts on these results.

Limitations and Future Studies

There were several limitations to this study affecting the interpretation of results. First, this study had a small sample size of only 19 athletes. As mentioned previously, this study was part of a larger study, the Athlete Study, an ongoing study with many more participants than were included in this analysis. However, at the time that this particular analysis was done, only 20 athletes had completed hair samples for cortisol analysis at all three timepoints. Of these 20, 1 athlete was deemed ineligible and removed from this analysis due to a hormonal IUD, which is known to affect cortisol due to a more sensitive cortisol responsivity (Aleknaviciute et al., 2017), potentially skewing results. In addition, a few athletes still had not completed all appointments

for either pre- or off-season and were therefore missing data points, further limiting data. Repeating this study with a larger sample size could help to normalize data and make the results more generalizable to the collegiate athlete population.

Another limitation to this study was the heterogeneity of sports included. On one hand, including several different sports rather than limiting it to just one sport or sport type (i.e. running or endurance sports) (Skoluda et al., 2012) makes the analysis more applicable and generalizable to a wider population of collegiate athletes, and more generally analyzes the effects of stress common to all athletes on cortisol exposure. However, this heterogeneity also lent itself to considerable variability in aspects such as training volume and time in between season phases, possibly impacting the results. As mentioned previously, we chose to analyze hair cortisol as a reflection of metabolic, physical, and psychological stress within the same season phase rather than as a reflection of stress in the previous season phase. A 3 cm hair sample represents cortisol exposure in the previous 3 months (Kirschbaum et al., 2009). For athletes whose hair samples were taken less than 3 months (90 days) apart, hair cortisol would theoretically capture stress in the previous season phase to each sample taken. However, for athletes whose hair samples were taken more than 3 months apart, hair cortisol would more likely capture stress within the same season phase. Our rationale only took into account average time between seasons for all athletes for simplicity, but this ignores a wide range of variability between individuals. A future analysis taking into consideration this variability and analyzing cortisol exposure more tailored to the season phases of each sport would likely obtain more accurate results and could even show us a completely different picture than the results of the present study.

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