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UNIVERSITY OF CALGARY

CHESS: Changes in Hormones with Exposure to Student Stress

by

Laura Elizabeth Crack

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

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Abstract

Salivary cortisol levels measure the acute stress response and this daily measurement occurs in two phases: the cortisol awakening response (CAR; 30 minutes after waking) and the diurnal cortisol response (the slope of the trend line associated with the remaining periodic samples throughout the day). Progesterone, one of two female sex hormones associated with the menstrual cycle (MC) is not well documented in terms of the stress cycle. The primary objective of the CHESS study was to prospectively investigate the impact of chronic stress (measured by the Student-Life Stress Inventory) on salivary cortisol and progesterone levels among female undergraduate students (N=19), while controlling for MC phase. Participants displayed blunted CAR, possible hypothalamic-pituitary-adrenal axis dysregulation manifested in irregular diurnal cortisol patterns, and changes in progesterone levels in response to academic stress. This finding provides a foundation for future studies to examine the relationship between cortisol and progesterone during times of stress.

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Dedication

This thesis is dedicated to:

Coach Ron Richards

Who has pushed me towards athletic, academic and life success for 13 years.

And to:

Guillaume Lévesque

Who taught me to slow down and enjoy the view along the way.

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List of Symbols, Abbreviations and Nomenclature

ABBREVIATION	DEFINITION
BMD	Bone Mineral Density
CHESS	Changes in Hormones with Exposure to Student Stress
CL	Dr. CM Lebrun
COC	Combined Hormonal Oral Contraceptive
DG	DO Garcia
ECG	Electrocardiography
EH	Exogenous Hormone
FSH	Follicle Stimulating Hormone
GNRH	Gonadotropin-Releasing Hormone
GLTEQ	Godin Leisure-Time Exercise Questionnaire
HPL	Human Performance Lab
HR	Heart Rate
HRV	Heart Rate Variability
IUD	Intrauterine Devices
JM	Dr. JM Murias
LH	Luteinizing hormone
LC	LE Crack
MC	Menstrual Cycle
MD	Medical Doctor
NCHA	National College Health Assessment
NH	Non-Exogenous Hormone
PPG	Photoplethysmography
RC	Research Coordinator
RS	RE Stokes
SI	SL Illingworth
SSI	Student-Life Stress Inventory
U OF C	University of Calgary

Chapter 1 Introduction

1.1 Purpose

The CHESS (**C**hanges in **H**ormone with **E**xposure to **S**tudent **S**tress) study was designed to investigate the physiological impacts of long-term student stress. The study prospectively observed perceived self-reported stress levels, (measured by the Student-Life Stress Inventory questionnaire), among female undergraduate students at the University of Calgary, and objectively measured cortisol (both the awakening response and diurnal daily levels) and progesterone levels across several menstrual cycles, throughout the academic year. The purpose was to investigate the relationship between perceived stress and measured hormonal stress levels and to explore the mechanistic relationship between cortisol and progesterone within the stress response, as it is unclear in existing literature.

1.2 University Student Stress

The National College Health Assessment (NCHA) recently reported that a fifth of Canadian postsecondary students were depressed, anxious or battling other mental and general health issues, such as lack of sleep (Chiose, 2016). Between 2013 and 2016, the number of surveyed students who reported being in good overall health decreased by eight percent (Chiose, 2016). In Canada, both media reporting and published research highlight increasing stress levels among university students as a widespread problem affecting academic performance and quality of life (Chiose, 2016).

Stress is considered one of the most prevalent risk factors for decreasing mental health among university students due to a large range of academic, social, and personal challenges that

these students must face daily. Academic challenges associated with university life are obvious and common to most students and include: coursework, assignment deadlines, examinations and peer competition (Hamilton, 2006), while personal challenges occurring throughout the university time period are often less conspicuous and more variable between students. Personal stressors can range from daily hassles or frustrations (which may include financial difficulties and/or lack of access to necessary resources) to relationship difficulties and social pressures (Gadzella, 1994; Hamilton, 2006). The process of obtaining an undergraduate university degree is unique to each student and many students are faced with unusual challenges (Drake, 1991).

The University of Alberta surveyed undergraduate students (N=457, 63% female, 37% male) to determine their stress levels and 71% reported their lives as either stressful or very stressful, while only 16.6% reported acceptable stress levels (Campbell, Svenson, Jarvis, 1992). In this group of students, females were more likely to report an unacceptable level of stress than males, with female students over 22 years of age reporting the highest levels of stress overall (Campbell, et al., 1992). More recently in 2013, 86.9% of U of A students reported having felt overwhelmed by all they have had to do (*Student Mental Health at the University of Alberta: an Overview*, 2015). Female students also tend to report unacceptable stress levels earlier in the academic semester and maintain these high levels of stress for a longer duration of the semester when compared to their male counterparts (Doyle-Baker, Verge, McClelland, Fung, 2018). Students not only feel stressed, but they are also exposed to an environment of peer pressure that influences their coping mechanisms for handling the stress. These coping mechanisms likely include unhealthy or maladaptive behaviours (Böke, Mills, Mettler, & Heath, 2019), which may contribute additively to their already high stress levels, leading to a vicious cycle negatively

affecting both physical and mental health. Student success may also be impacted by stress via withdraw from student engagement and learning (Ramey, Lawford, Chalmers, & Lakman, 2019).

Based on this reporting, female students are more likely to be affected day-to-day by the perceived "feelings" associated with stress, however despite this sex difference, stress and mental health is an important topic for all students. There are many psychological, sociological and physiological factors that may be contributing to the stress level discrepancy between males and females. One key difference between the sexes is hormonal physiology. Therefore, the CHESS study focused on the physiological response associated with stress among female university students.

1.3 A Brief Overview of the Physiological Stress Response in Females vs Males

All determinants of human health are sex-dependent to varying degrees and stress is no exception. Mechanisms of the hypothalamic-pituitary adrenal (HPA) axis, as well as sexual development (especially puberty and menopause) exacerbate the differences between males and females. Two hormones involved in the stress response in both sexes are cortisol and progesterone (Herrera, Nielsen, & Mather, 2016). Cortisol is most commonly associated with metabolic control during periods of stress, and is responsible for the mobilization of glucose to prepare the body to handle stressful situations, referred to as the fight or flight response. Progesterone is generally associated with its role as a female sex hormone involved in the menstrual cycle, reproduction and pregnancy. Despite this stereotype, progesterone is also a precursor to corticosterone. Animal model research has shown that the adrenal glands in both sexes release progesterone in addition to cortisol, during the response to stress (Herrera et al., 2016; Hueston & Deak, 2014). The main difference in the physiological stress response between

sexes lies within the large discrepancy in the bioavailability of resources to release cortisol and progesterone during exposure to stress. While a male has the same bioavailability at all times, the cyclical nature of female hormonal fluctuations associated with the menstrual cycle impacts this daily bioavailability. These mechanisms are further discussed in the literature review chapter of this thesis. This pattern of hormonal fluctuation makes the female physiological stress response distinctly different from the male stress response and this area in the literature is underexplored (Hueston & Deak, 2014). This mechanism in combination with the findings that female students report higher stress levels than their male counterparts informed the CHESS study. There are likely other psychological and sociological sex-dependent factors at play, however these are not addressed in this study.

1.4 Defining Stress

Stress has many definitions because it is an abstract concept and it crosses several scientific areas including the domains of the medical and social sciences. One of the more general definitions is “a real or interpreted threat to the physiological or psychological integrity of an individual that results in physiological and behavioural responses” (Balhara, Verma, & Gupta, 2012). This definition encompasses both the psychological and physiological aspects of stress, which aligns with the CHESS study’s primary purpose of identifying the relationship between self-reported perceived stress levels (psychological) and the physiological responses to these stressors, among female students over an academic year. Acute stress occurs because of exposure to a short-lived or temporary negative situation. It can be physical, emotional or psychological in nature, but allows a quick and complete recovery of the physiological changes. Whereas, chronic stress exists due to a long-lasting state during which one cannot fully recover

physiologically (Trevisi & Bertoni, 2009). In the literature review section, the tools used to quantify these psychological and physiological stress components are outlined.

1.5 Rationale and Population

Female university students are at a high risk of developing chronically elevated stress levels, and there is limited research exploring the long-term impact of chronic stress on female hormonal physiology. Due to the complexity of the menstrual cycle and this perceived barrier, women are generally underrepresented in health and sports science research (Bruinvels et al., 2016; Cislak, Formanowicz, & Saguy, 2018; Holdcroft, 2007). The CHESS study observed stress levels, and the associated physiological changes of stress among the female undergraduate population at the University of Calgary (U of C). The U of C is a large institution in western Canada, comprised of over 30,000 students, located in an urban center with approximately 1.5 million residents ("World Population Review," 2019) and this sample population is likely to be representative of a large post-secondary institution in an urban region. This study focused on contributing to the understanding of overall student health and wellbeing in Canada and provides a foundation for investigating the impact of student lifestyle on long-term health risk factors.

In addition to the primary purpose of the CHESS study, our secondary findings shed light on categories, or types, of stress that most strongly contributed to total stress scores in this sample population. The questionnaire employed in this study (see Chapter 2) to evaluate self-reported perceived stress levels used categories related to type of stress, so as to identify which types were perceived most often within the population. Furthermore, providing insight into the timing of the fluctuation of types of stressors contributing to overall stress throughout an academic year. This information may be useful in tailoring some of the health and wellness

services at universities by providing information on the types and timing of support services needed to improve student wellbeing. For example, at the beginning of the semester, when changes are potentially causing increased levels of stress, university student services could provide workshops educating students on how to both anticipate and manage times of stressful change. The larger CHESS study also entailed data collection and investigation of Heart Rate Variability (HRV) and breakfast eating habits in response to student stress across the MC, but these objectives will not be assessed within this thesis.

1.6 Objectives

The primary objective of the CHESS study was to determine if there was an association between self-reported stress levels, measured by the Student-Life Stress Inventory (SSI) scale, and salivary cortisol and progesterone levels during the menstrual cycle over an academic year. The secondary study objective was to determine the relationship between baseline progesterone levels and the magnitude of cortisol change during the acute stress response.

The study protocol allowed us to compare progesterone and cortisol levels in response to academic stress and to assess the relationship of these hormones to determine if one hormone influences or mediates the magnitude of change in the other over an academic year. If women have been experiencing an adrenal release of progesterone in response to stress (as outlined in Chapter 2: Literature Review), then this may be contributing to a pattern of greater bioavailability of cortisol in response to stress during the high progesterone phases of the menstrual cycle (Kirschbaum, Kudielka, Gaab, Schommer, & Helhammer, 1999). Our goal was to help clarify the role of progesterone and its interaction with cortisol during the human stress response in a population known for its high reported stress levels.

1.7 Hypotheses

The primary hypothesis was: There will be a linear correlation between self-reported stress levels and salivary cortisol and progesterone levels throughout the academic year. Self-reported stress levels would increase from the beginning of each academic semester until midterm exams and remain elevated until finals and subsequently cortisol and progesterone levels would increase and plateau due to their previously identified co-involvement in the physiological stress response. Although cortisol's involvement is well documented, some research suggests that progesterone increases with stress, while other research suggests it decreases or shows no significant relationship (Gaffey & Wirth, 2014). This is discussed in greater detail in the literature review throughout Chapter 2.

The secondary study hypothesis: There will be a significant correlation between baseline progesterone levels and the magnitude of the cortisol change in response to academic stress. Participants with naturally higher levels of progesterone prior to a stressful academic time period (ex. Final exams), would exhibit a greater change in cortisol with response to increased stress. Previous literature suggested that increased progesterone (either natural or synthetic) before stress lead to a greater output of cortisol in response to stress (Roca et al., 2003). Both natural menstrual cycle fluctuations as well as administered medications containing synthetic progestins likely impact the daily stress response in females. The CHESS study results contribute to clarification of this this complex relationship.

Chapter 2 Literature Review

2.1 Literature Search

A literature search was conducted with the guidance of a Kinesiology Librarian (AH) regarding hormones involved in the physiological stress response. The Medline database was used with the mesh terms: stress* and cortisol* resulting in 16,798 results and a second search conducted using the terms: stress* and progesterone produced 2,436 results. These searches were narrowed to three terms: stress* and cortisol* and progesterone*. This search produced 361 results and after reviewing each title and abstract for exclusions 14 articles were identified. Articles were excluded if they related to pregnancy, specific diseases, drug use, addiction, or if they were not related to the physiological stress response.

A separate search related to stress levels among Canadian university students was conducted using Google Scholar with the terms: university student stress in Canada. The "Grey literature" included non-scientific reporting from Statistics Canada and newspapers such as The Star and The Globe and Mail. These were used to identify self-reported stress levels, specifically, among Canadian university students.

2.2 The Impact of Stress on the Human Body

Stressors come from a variety of sources, but as outlined in the definition of stress, regardless of the context of the stressor, the body is exposed to a perceived threat or challenge and the resulting physiological stress response is the same in all cases (Anderson, Litzenberger, & Plecas, 2002). This response commonly known as "Fight or Flight" was highlighted during the 1930s as a part of basic research in psychology (Cannon, 1936). The fight or flight process is

generally associated with immediate, acute bodily changes; however, fundamental stress research shows that chronic or long-term exposure to stress elicits a prolonged fight-or-flight response (Selye, 1956).

The short-term physiological reaction to stress involves the autonomic nervous and endocrine systems (Silverthorn, 2015) controlled by both the upper and lower cortices of the brain. The higher centres are responsible for consolidating the sensory information related to the stressor by comparing it to previous experiences which helps determine whether or not there is a stressful situation at hand. The lower centre controls the physiological alterations needed to cope with the stressor. The alterations characterizing this response are driven by the sudden release of catecholamines and subsequently, glucocorticoids. The body responds to these chemical messengers by increasing physiological arousal and alertness associated with heart rate, breathing rate and body temperature. The digestive system is shut down and blood is diverted away from other internal organs, while glucose is mobilized to provide the energy to the high priority skeletal muscle. Platelet aggregation increases to help defend against injuries that may be encountered as a result of the stressor (Anderson et al., 2002). The common goal of all these stress response components is to prepare the body to handle the stressful situation. In the moment, the mobilization of energy to the skeletal muscles will take priority over all other bodily functions. This is a fundamental biological process, key to survival, but complications may arise when this process becomes more chronic than acute. In other words, constant exposure to stress can cause this fundamental biological process to elicit harm on the body.

The immune response as alluded to above is also involved as antibody production slows and the circulation of white blood cells is reduced as bodily priorities shift during stress. This helps reach the common goal of survival in the short-term, but if the stress response is chronic or prolonged, it leads to suppression of the immune system (Anderson et al., 2002). This is why high stress over extended periods of time is often associated with illness in “lay science” reporting. Other common associations with long-term stress are: decreased memory, learning, cognitive function, sleep and burnout (Anderson et al., 2002). Although these symptoms may not always be life threatening, they have an impact on daily functioning and quality of life.

2.3 Cortisol

2.3.1 Cortisol

Cortisol is a steroid hormone (molecular formula $C_{21}H_{30}O_5$), within the glucocorticoids class of hormones, and is made in the cortex of the adrenal glands and released into the blood. Normal bodily levels of cortisol produce a diurnal curve including peak levels approximately 30-minutes after waking in the morning, known as the cortisol awakening response or CAR, and a negative slope throughout the day. The cortisol fluctuation, however is extremely acute throughout the day, and variations to the diurnal curve are often observed (Clow, Hucklebridge, Stalder, Evans, & Thorn, 2010; Stone et al., 2001).

2.3.2 Measurement of Cortisol

Cortisol is typically the primary biomarker indicative of the stress response (Hellhammer, Wüst, & Kudielka, 2009) in both animal and human research models. Cortisol is a glucocorticoid associated with an important hormonal response system to stress – the hypothalamic-pituitary-

adrenal (HPA) axis (Herrera et al., 2016). It can conveniently be measured in saliva where its secretion pattern mimics concentrations in circulation (Lippi et al., 2009).

2.3.3 Cortisol Response to Stress

The physiological role of cortisol is to mobilize energy for use during times of stress in preparation for the fight-or flight response. The acute response to stress elevates cortisol for approximately 45-60 minutes (Clow, Patel, Najafi, & Hucklebridge, 1997). Many types of stressors have been reported to increase cortisol excretion in humans such as physical threats, social stress, isolation and rejection, even though not all types of stress require a drastic increase in mobilization of energy to manage them (Gaffey & Wirth, 2014). Repeated or long-term exposure to stress without return to homeostasis can induce chronically high cortisol levels (Trevisi & Bertoni, 2009), commonly referred to as hypercortisolism. If managing these stressors does not require utilization of the energy mobilized, excess gluconeogenesis leading to hyperglycemia or high blood sugar may result. Furthermore, hypercortisolism is associated with muscle and fat breakdown from stores, followed by fat deposits in the trunk. These symptoms are risk factors associated with other chronic diseases, including Type 2 Diabetes (Silverthorn, 2015). Therefore, long-term exposure to stress can contribute to chronic health problems.

Cortisol levels should peak in the morning approximately 30-minutes after waking (de Weerth, Zijl, & Buitelaar, 2003; Fries, Dettenborn, & Kirschbaum, 2009; Kudielka, Federenko, Helhammer, & Wüst, 2006) and drop throughout the day. Optimal salivary levels at each time interval include: 1) CAR: 14-25nmol/L, 2) Lunchtime: 5.0-10.0 nmol/L, 3) Evening: 2.0-5.0 nmol/L and 4) Bedtime: 1.0-4.0 nmol/L (Doctors Data Labrix, 2019).

Chronic stress may impact the HPA axis's ability to self-regulate the diurnal cortisol pattern (Wirth et a., 2011) influencing this diurnal curve by leading to higher than optimal cortisol values at all time points throughout the day. Chronic fatigue leads to an elevated CAR value and a steep negative daily slope with below-optimal levels during the three other measures. Finally, burnout has been associated with a blunted CAR and sub-optimal cortisol levels throughout the day (Laboratory ZRT, 2019). Each type of diurnal cortisol variation can lead to different symptoms and lower quality of life, overall.

Much research to date has investigated habituation of the cortisol response through repeated exposure to the same stressor because chronic exposure to stress and hypercortisolism may impact health and quality of life. Habituation would theoretically occur if a distinct stressor induced a given cortisol response in a subject upon the first exposure, followed by a reduction in future cortisol output in the same individual when responding to the same stressor. Selye's 1956 fundamental research in the field examined habituation via his "stages of resistance." He determined that repeated or continuous long duration stress will instigate the individual's coping mechanisms for adaptation. While these coping mechanisms tend to somewhat alleviate the emotional reaction, the overproduction of glucocorticoids persists, (Selye, 1956) demonstrating that although the emotional habituation is possible the physiological habituation is not. Mixed findings, however on this topic have been presented since Selye's fundamental work. Kirschbaum and collagues conducted a study using the Trier Social Stress Test and attempted to determine whether habituation to a single social stressor would lead to a reduction in cortisol excretion in the subsequent exposures (1995). Twenty healthy, non-smoking, male students at the University of Trier, with a mean age of 22.4 years, were recruited for the study. On five

different testing sessions, the subjects participated in a public speaking task and an arithmetic task in front of an audience to induce social stress. The details of the tasks varied slightly each session, but the principal remained the same. Salivary samples were taken at baseline, after the explanation of the task, and following the task completion. The sessions took place at the same time each day to control for circadian changes in cortisol levels. Cortisol peaked in the saliva collection ten minutes after finishing the stressful task and the mean cortisol level of all participants rose significantly from baseline to this time point across all five testing sessions, suggesting no significant habituation effect. The subjects were subsequently grouped into high responders and low responders based on their cortisol levels. High responders showed a significant increase in cortisol levels over all five sessions, while low responders only had a significant increase on the first day, and did not elicit a significant increase during the subsequent four trials (Kirschbaum et al., 1995). These results suggest that low responding individuals are able to physiologically adapt to stressors with repeated exposure over time, reducing the level of their hormonal stress response, while high responders do not display this ability to habituate. This causes a positive feedback in the high responders, leading to chronically elevated cortisol, and potentially higher risk of future adverse health effects, such as the associated risk of cardiovascular disease (Krantz & Manuck, 1984).

2.3.4 Cortisol Response to the Menstrual Cycle

Cortisol is typically the primary biomarker of stress, as previously stated. This usage of cortisol as a measurement tool, however requires a thorough understanding of its normal longitudinal variability, which is limited in research related to women. It is assumed, for example, that basal cortisol profiles do not vary across the menstrual cycle (MC). McCormick et al., (2001) and Liening et al., (2010) both detected no difference in salivary samples of cortisol

over the course of the menstrual cycle. Furthermore, Nepomnaschy et al., (2011) attempted to determine if cortisol excretion was independent of the day of the MC through a longitudinal study. Their results showed no significant variation of cortisol levels throughout the days of the cycle in subjects who had a regular 28-day cycle. However, if either the follicular phase or the luteal phase was greater than 14 days, levels of daily cortisol excretion were impacted (Nepomnaschy, 2011). Although the MC is often regarded as 28 days in duration, many women do not have a 28-day cycle (Gandara, Leresche, & Mancl, 2007), and if cortisol does follow a time-dependent pattern during longer MCs, ignoring this cyclic variation could lead to erroneous attributing of physiologic stress (Nepomnaschy, 2011). The assumption that basal cortisol levels are stable across the MC is based on conflicting evidence, and, because of this discrepancy in the literature, we cannot confidently state that there is no fluctuation of cortisol during the MC (Liening, Stanton, Saini, & Schultheiss, 2010; McCormick & Teillon, 2001). The MC therefore, should be considered in future research regarding the physiological stress response in females.

2.4 Progesterone

Progesterone is a steroid hormone with a similar molecular formula ($C_{21}H_{30}O_2$) to cortisol and is involved in the female reproductive process (Dinny Graham & Clarke, 1997). It is one of the primary female sex hormones associated with MC hormone fluctuations. Clinically normal salivary levels of progesterone range from 127-446 pg/mL (Doctors Data Labrix, 2019). The ratio of estrogen to progesterone is also of clinical importance and must reach a minimum of 200 in order to be considered progesterone sufficient, regardless of the salivary progesterone level (Doctors Data Labrix, 2019).

2.4.1 Progesterone and Hormonal Fluctuations of the Menstrual Cycle

The MC occurs due to natural fluctuations of sex hormones in females and every woman experiences a unique MC. As stated above the typical MC lasts 28 days spanning the follicular, ovulation and the luteal phases, so as to achieve three physiological roles: to bring an ovum to maturity, to replenish the uterine tissue that will be needed for growth of the fetus and to prepare the uterus for pregnancy (Silverthorn, 2015). The four main structures associated with the menstrual cycle are: the hypothalamus, pituitary gland, ovaries and uterus, which use eight hormones as messenger signals, namely: gonadotropin-releasing hormone (GnRH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), estrogen, progesterone, prolactin, oxytocin and prostaglandins (Silverthorn, 2015). Of these, estrogen and progesterone are most widely used to characterize the menstrual cycle length and phases. The onset of menses, usually reported as day 0 or day 1 of the cycle, is characterized by low levels of estrogen and progesterone and marks the beginning of follicular phase. Estrogen then rises to its peak in the late follicular phase approximately 24-46 hours before ovulation (Mihm, Gangooly, & Muttukrishna, 2011). Ovulation falls near the mid-point of the 28-day cycle. Approximately one week after ovulation, progesterone reaches its peak, and estrogen reaches its second highest concentration of the cycle (Silverthorn, 2015). This is known as the mid-luteal phase. If pregnancy is not achieved by this time point, menses occurs and the cycle repeats itself. Figure 2.1 (below), outlines the hormonal production and fluctuation of the menstrual cycle, which will be further explained in relation to the stress response within this chapter.

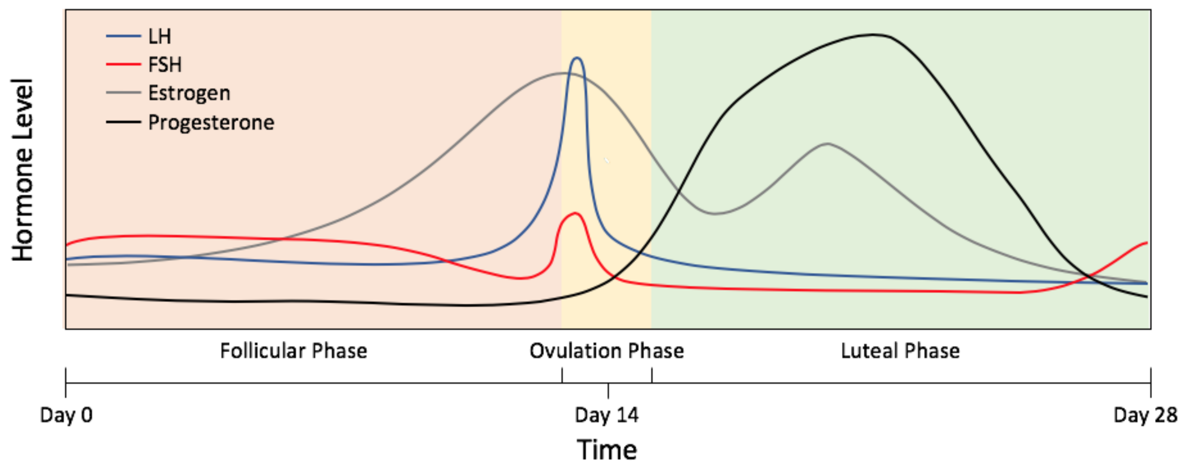

Figure 2-1 Hormonal fluctuation of the menstrual cycle

2.4.2 Progesterone Response to Stress

Research has shown that the adrenal glands in animals of both sexes release progesterone in addition to cortisol, during the response to stress (Herrera et al,, 2016). A number of studies using animal models as well as male subjects support these findings (Breier & Buchanan, 1992; Brown, Courtney, & Marotta, 1976; Cooper, Evans, Cook, & Rawlings, 1995; Deis, Leguizamon, & Jahn, 1989; Duncan, Knapp, Carson, & Breese, 2011; Elman & Breier, 1997; Fajer, Holzbauer, & Newport, 1971; Romeo, Karatsoreos, & Mcewen, 2006). Cortisol and progesterone are both steroid hormones synthesized from the cholesterol derivative pathways, thus a concurrent increase in production of these hormones makes sense. Unlike cortisol which is only produced by the adrenal gland, progesterone can be detected in the brain where it originates as well (Purdy, Morrow, Moore, & Paul, 1991). Brown and colleagues examined progesterone levels in rodents and saw increased levels of progesterone during exposure to multiple types of stressors (Brown et al., 1976). However, there has been mixed results in humans with respect to the involvement of progesterone in the stress response (Wirth, Meier, Fredrickson, &

Schultheiss, 2007). Gaffey et al. (2014) examined both levels of cortisol and progesterone in reaction to social and rejection stressors among men and women. Participants were randomly allocated into four possible groups: two groups acting as the stressed samples and two as the controls. The social stress group was informed that they must present a speech for evaluation, while the applicable control group had to write a non-evaluated essay. The rejection stress group played a computer game designed to make the participant feel ostracized, while the control group played a computer game designed to be inclusive. Salivary samples of cortisol and progesterone were taken both before (baseline) and after intervention to analyze the differences among participants in all four groups. To obtain useful salivary samples, participants were instructed not to eat or drink, brush their teeth, or partake in vigorous physical activity for two hours prior to the sampling. Although the rejection stressor activity did not elicit an increase in either cortisol or progesterone, the social stressor activity caused a significant increase in cortisol, but not progesterone. This result highlights the inconsistent findings of the role of progesterone in the human physiological stress response (Gaffey & Wirth, 2014).

Limited research has focused specifically on the impact of progesterone fluctuation with stress in eumenorrheic, naturally cycling human females. A recent study has shown that in response to an acute physical stressor (cold pressor test), no change in estradiol levels were detected, yet increases in progesterone and cortisol were observed (Herrera et al., 2016). The levels of estrogen, considered to be the most recognized hormone used to track the MC remained unchanged, while progesterone, often not measured or even monitored in MC research, may be fluctuating. This could potentially result in undetected changes to phase lengths within the cycle. Reporting of high progesterone levels have been previously associated with mental health

changes, including decreased informational processing, decreased verbal memory function and increased reported levels of fatigue (Freeman, Weinstock, Rickels, Sondheimer, & Coutifaris, 1992). Monitoring progesterone levels along with cortisol, may be a useful biomarker for identifying high stress levels, however further investigation needs to be completed to clarify inconsistencies across the existing and limited research.

2.4.3 The Menstrual Cycle (MC) During Stress

Stress plays a role in suppressing the functioning of the hypothalamus, which controls the pituitary gland and this, in turn, controls the thyroid and adrenal glands. Constant and/or excessive stress results in secretion of the adrenaline and cortisol hormones. Adrenaline increases energy and cortisol increases brain function and slows or stops nonessential bodily functions. Stress, therefore can interfere with the normal, timed and regular release of gonadotropin, a hypothalamic hormone. Given that both estrogen and progesterone release are products of a negative feedback loop involving the hypothalamus and pituitary glands, in general terms, the MC is likely impacted by stress (see Figure 2.2).

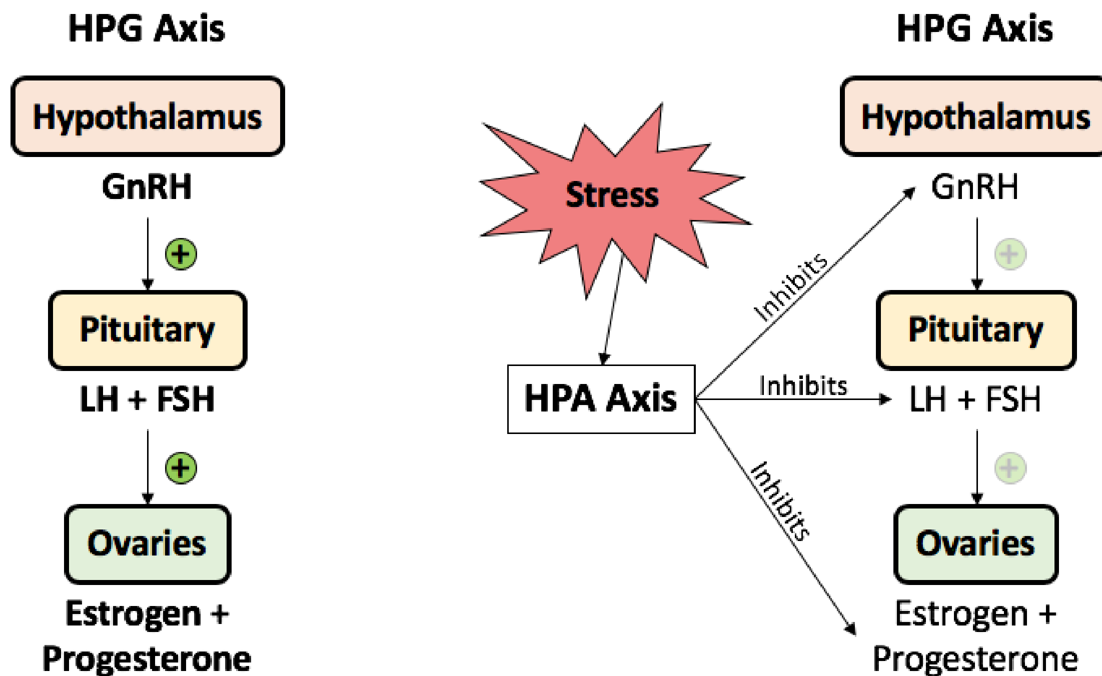

Figure 2-2 The mechanistic impact of stress on hormone production within the HPA axis.

Progesterone levels naturally increase in the mid-luteal phase of the MC and the literature identifies an increase in progesterone with exposure to stress (Herrera et al., 2016). This suggests that in addition to the natural mid-luteal increase in progesterone, exposure to stress may impact the cycle of progesterone in one of two ways, by: 1) causing a significant increase of progesterone during other phases of the cycle (possibly shortening the follicular phase or ovulation), or 2) magnifying the natural increase of progesterone during the mid-luteal phase to supra-normal levels.

Other literature suggests that progesterone levels decrease in response to stress, which adds confusion to this concept (Osakue, Onyenekwe, Ahaneku, Onyegbule, & Okunoghae,

2015). These findings appear largely dependent on the time of measurement in relation to the event of stress. Osakue et al. (2015) reported lower levels of progesterone in the time period leading up to a stressful event, in which anticipatory stress may be occurring. Because stress is not a black and white concept, physiological responses may differ depending on the type of stress and the time of measurement in relation to the stressful event. Furthermore, many studies do not consider that progesterone is released from two sources in females: the adrenal and the ovaries. While stress may be inducing an increase in progesterone from one source, it could simultaneously be supressing the other source, leading to the discrepancy in findings.

2.5 Cortisol and Progesterone Relationship

The relationship between cortisol and progesterone remains under mixed findings. Liening et al. (2010) saw a positive correlation between cortisol and progesterone, yet also stated that MC phase affected progesterone, but not cortisol. It is unclear how these two findings coexist. Wirth et al. (2007) examined the correlation between cortisol and progesterone in 373 participants. All collection sessions took place at the same time of day to reduce the confounding effect of circadian rhythm on the hormones. Results showed that cortisol and progesterone were positively correlated in men as well as in women who were using hormonal contraceptives, but not in naturally cycling females (Wirth et al., 2007). In the latter group, a regression of cortisol on progesterone was plotted using elapsed time as a self-reported cycle day. Although it is important to consider that self-report of the MC day is not the most controlled form of measure, results remained unchanged and no significant correlation between cortisol and progesterone was detected using the regression in this sample group (Wirth et al., 2007). Kirshchbaum et al. (1999) conversely found that naturally cycling women in the luteal phase and men, when exposed to

social stress, resulted in comparable cortisol levels. Women on oral contraceptives or naturally cycling in the follicular phase exhibited significantly lower salivary free cortisol levels (Kirschbaum et al., 1999). This suggests that in females, lower levels of progesterone are associated with lower levels of unbound cortisol via naturally lower progesterone of the follicular phase and oral contraceptive progesterone suppression.

These contradictory study findings demonstrate that there is likely an involvement of progesterone, along with cortisol, in the stress response based on the correlation between the two hormones, however the relationship remains unclear due to discrepancy in findings. Interestingly, a study by Roca et al. (2003) showed that women administered synthetic progesterone exhibited a larger increase in cortisol in response to physical stress than those women administered estrogen, suggesting a correlation between baseline progesterone levels and the magnitude of the cortisol increase in the physiological stress response. However, in this experiment subjects were limited to women with induced hypogonadism, limiting the generalizability of the results (Roca et al., 2003).

Herrera et al. (2016) investigated the relationship between cortisol and progesterone levels in response to an acute stressor. Mediation analysis determined that the two hormones were not only positively correlated, but the level of baseline progesterone influenced the magnitude of the cortisol change from baseline to post-stressor (Herrera et al., 2016). Thus, higher baseline progesterone levels induced a larger change in cortisol levels. These findings show that the relationship between the two hormones in the stress response is complex and warrants further investigation. Since some research studies choose to measure progesterone

values across the MC, but only report an average value (Nielsen, Ahmed, & Cahill, 2015; Petersen, Kilpatrick, Goharzad, & Cahill, 2015), this relationship is important. Performing analyses using average progesterone values will not effectively examine the impact of daily progesterone levels on the cortisol response. This approach may lead to erroneous attributions of increased stress related to a larger amount of free cortisol, when it is also possible that increased progesterone level at the time of the salivary collection was the cause of the increase in free cortisol.

2.6 Oral Contraceptives and Stress

2.6.1 Mechanisms of Hormonal Birth Control Methods

Female underrepresentation in health research, according to Sims and Heather, (2018) is driven by the cyclic variation in hormones associated with the menstrual cycle, but is further complicated by the mechanisms of hormonal contraceptive methods, which are often ignored in research protocols. To effectively integrate hormonal contraception into the CHESS study, basic mechanistic functions of birth control were assessed. Rivera and colleagues provide a brief outline of the mechanisms of different types of birth control readily available for public use (Rivera, Yacobson, & Grimes, 1999). The most well-known type of birth control is "the pill" or the combined hormonal oral contraceptive (COC), which has become a notable milestone in the history of public health (CDC, 1999). There are two types of COCs: 1) Contemporary COC and 2) Monophasic COC. Contemporary COCs (including biphasic and continuous COCs) contain a combination of ethinyl estradiol and one of many possible presentational agents, derived from testosterone, progesterone and/or spironolactone (Dragoman, 2014). This type of COC varies in dosages of either the estradiol or progestin components over the active cycle "to reduce overall hormonal exposure and to more closely mimic a normal cycle" (Dragoman, 2014). Monophasic

COCs give a constant dose of estrogen and progestin components throughout the active cycle. This alleviates some complications resulting from incorrect usage of the medication because all active pills are the same, but strays away from the natural hormonal fluctuations of the cycle. Both types of COCs are administered for 21 consecutive days followed by 7-days without pills (or with placebo pills). The primary function of COC's is to prevent ovulation, achieved by inhibiting pituitary production of FSH and LH (Dragoman, 2014), two MC hormones which would otherwise increase at the ovulation time-period. This is achieved via pituitary inhibition, suppressing the normal production of GnRH, subsequently indirectly reducing FSH and LH. Although this suppression of ovulation is possible with administration of either estrogen or progestin alone, they work better in combination as the progestin components are more effective at blocking the LH production, while the estradiol components helps maintain a regular "cycle" that actively sheds the endometrial layer at desired (or more standard) intervals (Rivera et al., 1999).

In addition to the COC, there are newer vaginal ring contraceptives, which release etonogestrel/ethinyl estriol into the vagina. For this method, a ring (54mm in diameter) is inserted into the vagina and remains there for a period of three weeks. The ring is removed for the 7-day bleeding period and a new ring is subsequently inserted (Szarewski, 2002). The vaginal ring, more commonly known as the NuvaRing delivers 0.12mg of etonogestrel and 0.015mg of ethinyl estradiol per day (FDA, 2002). The intrauterine device (IUD) is another inserted birth control method and there are two main types the: copper IUD or hormonal IUD. The copper IUD is designed to prevent fertilization by blocking sperm or reducing sperm motility, thereby stopping them from reaching the Fallopian tubes (Rivera et al., 1999; Stanford & Mikolajczyk,

2002). It is possible for the sperm to get past the IUD and into the fallopian tubes, however, fertilization typically does not occur because the sperm have been damaged. (Rivera et al., 1999). Hormonal IUDs work by releasing synthetic progestins at the site of insertion to make the cervical mucus unfavourable for sperm transport and to suppress the endometrium. Although less hormonally significant than oral contraceptive methods, the hormonal IUD still alters overall levels of progestins in the body due to the synthetic progestin release in the cervix.

2.6.2 Impact of Hormonal Birth Control on Cortisol

Older literature has reviewed the impact of long-term hormonal birth control use on stress and cortisol levels. A variety of work in the 1980s and early 90s summarized by Kirschbaum et al. 1995, suggests that total blood cortisol levels are higher in OC users than naturally cycling women. The mixed evidence suggests that unbound cortisol levels detected in saliva samples are less affected by OC use, but still produce higher cortisol awakening levels than non-OC users (Kirschbaum et al., 1995). The study completed in 1995 at the University of Trier, used the previously established Trier Social Stress Test to examine the cortisol response between the two groups and clarify the existing literature. Results demonstrated that baseline stress levels and perceived (self-reported) stress levels as measured via questionnaire, were not significantly different between the two groups. All participants displayed an increase in cortisol levels 30-minutes post-stressor compared to baseline, but the control group's increase was significantly greater than the OC group's, suggesting a blunted response due to the hormonal birth control (Kirschbaum et al., 1995). Although there is some newer research in the field, the need still exists to clarify the mixed results in the literature.

2.7 Stress Measuring Tools

2.7.1 Self-Reported Stress (Psychological Measuring Tools)

Many measures of stress are typically obtained via self-report and although this can be subject to recall bias, these tools do have acceptable reliability for measurement of chronic stress. Pozos-Radillo et al. (2014) examined two scales for self-reporting stress applicable to university students: The Academic Stress Inventory (ASI) and the Stress Symptom Inventory (SSI). The ASI was validated by the Spanish Society of Anxiety and Stress (SSAS) and was successfully assessed for internal consistency (ICC 0.9). This assessment uses scaled answers in response to 11 situational stressors to determine a score of academic stress in the subject (Pozos-Radillo, Preciado-Serrano, Acosta-Fernández, Aguilera-Velasco, & Delgado-García, 2014). The SSI is also a self-report scale, and includes a list of 42 psycho-physiological symptoms used to assess chronic stress levels (ICC 0.94). This questionnaire has a classification of high, moderate and low levels of chronic stress; high is two to three standard deviations above the mean, moderate is one standard deviation above or below the mean, and low is two to three standard deviations below the mean (Pozos-Radillo et al., 2014).

The SSI used by Gadzella et al. (1994) is composed of a list of 51 stress items in 9 different categories: frustrations, conflicts, pressures, changes, self-imposed stress, physiological experiences, emotions, behaviours and cognitive appraisal to determine an overall stress level score. The questionnaire was developed to "reflect students' life experiences on and off campus" and was based on a theoretical model by Morris regarding types of stressors and reactions to stressors (Gadzella & Baloglu, 2001; Gadzella, 1994). Stressors are defined as circumstances interpreted as taxing to cope with, based on the capabilities of the subject in question (Feldman, 2012; Gadzella, 1991; Hockenbury & Hockenbury, 2010), while reactions to stressors are

behavioural, physiological, emotional or psychological responses to the stressors that the individual exhibits (Feldman, 2012; Gadzella, 1991; Hockenbury & Hockenbury, 2010). Based on this model, translating to the SSI, the 9 categories are separated into types of stressors (frustrations, conflicts, pressures, changes and self-imposed stressors) and reactions to stressors (physiological, emotional, behavioural and cognitive). The original intent of the questionnaire, was not to just determine an overall stress score, but to assess the relationship between types of stress and reactions to stress (Webb, 2012). The SSI and its subcategories have been tested for reliability and validity, with Cronbach's alphas for each category ranging from 0.52 (frustration category) to 0.85 (change category) and an overall questionnaire score of 0.78. Based on Nunnally (1978), a minimum Cronbach's alpha of 0.7 is required to demonstrate internal consistency, and on average the SSI is meeting this minimum in most categories. The questionnaire was repeated twice in a 3-week period and the results were compared resulting in Pearson product-moment correlation of 0.78 (Gadzella, 1994).

The SSI questionnaire uses a 5-point Likert scale, rating each of the 51 items from 1-5 (1 being least applicable, 5 being most applicable). The total inventory score is determined by: summing items 1-49 and adding to the sum of the inverse of the scores from items 50 and 51. The two subscales, types of stressors and reactions to stressors, are also computed from the questionnaire. A score for types of stressors is calculated by summing items 1-23 and reactions to stressors is computed by summing items 24-49 and adding the inverse of items 50-51 (Webb, 2012).

2.7.2 Cortisol Levels (Physiological Measuring Tool)

Stress levels can be measured through blood and or salivary samples of hormone levels (Gaffey & Wirth, 2014). High amounts of glucocorticoid hormones, known to be present during the physiological stress response, can help detect stress levels in individuals. Measuring cortisol in the blood plasma is often employed in animal model research (Sumpter, Dye, & Benfey, 1986), some human research (Kirschbaum et al., 1995) and in clinical practice. Active free cortisol measured in saliva has been shown to have the same diurnal rhythm as serum cortisol, one that typically declines rapidly throughout the waking day (Heinrichs, Baumgartner, Kirschbaum, & Ehlert, 2003; Hellhammer et al., 2009; Inder, Dimeski, & Russell, 2012; Takai et al., 2004). However, it is common to see individual differences in the diurnal cortisol curve (Stone et al., 2001) and acute changes in cortisol throughout the day (Clow et al., 1997).

Saliva collection is simpler and less invasive than blood draws. In some cases, participants may find the blood draw itself stressful, potentially influencing the results in studies observing natural stress levels.

2.7.3 Progesterone Levels (Physiological Measuring Tool)

It has been previously established in animal models that progesterone is another hormone released during the stress response and therefore progesterone is a useful biomarker of stress as well. Similar to cortisol, progesterone can be measured via both blood plasma and saliva (Choe, Khan-Dawood, & Yusoff-Dawood, 1983). However, salivary samples are much more commonly used to measure progesterone in research related to the MC (Ellison & Lager, 1986; Liening, 2010) primarily because they are easy and non-invasive. Companies such as Labrix (OR, USA) and Salimetrics (CA, USA) have saliva collection kits that measure progesterone and cortisol and they are available for research purposes under the supervision of a physician.

2.7.4 Heart Rate Variability (Physiological Measuring Tool)

Heart Rate Variability (HRV) decreases in response to stress and according to a meta-analysis by Castaldo et al. (2015) it is also an indicator of change in stress level (Castaldo et al., 2015). HRV measures the variation between heart beats and has been used to assess the autonomic nervous system (ANS) in different diseases and under various conditions (Malik, Bigger, Camm, & Kleiger, 1996). In general, HRV is influenced by many several factors including chemical, hormonal and neural modulations, circadian changes, exercise, emotions, posture and preload.

There are many tools that can be used to measure HRV including a heart-rate chest strap, electrocardiography (ECG) and the more recently developed smartphone technology using photoplethysmography, or PPG (Shaffer & Ginsberg, 2017). PPG is typically paired with a smartphone application app, i.e. the HRV4Training app (Altini, 2013), and has been validated against the ECG results (Plews et al., 2017). There are typically two domains used to measure the various indices associated with HRV. The standard deviation of beat-to-beat intervals (SDNN) is used to determine HRV by measuring the time domain between R-R intervals and by comparing each, so that a total index of variation can be established (Koskinen et al., 2008). A second commonly used domain employs the root mean square of successive differences (rMSSD) between the R-R intervals to determine variation of vagal activity (Malik et al., 1996) because the innervation in heart is by the vagus nerve and this establishes normal sinus rhythm (Koskinen, 2008). All the aforementioned methods of HRV measurement have been validated (Plews et al., 2017) and recently the rMSSD has been used to detect ovulation (Kokts-Porietis, Minichiello, & Doyle-Baker, 2019). The HRV4Training app is a convenient and accessible tool

to use in all settings, lab and field, for day-to-day measurement and is often used for research purposes when measuring fatigue and general stress.

2.8 Weaknesses in the Current Literature

The existing literature related to the physiological stress response as a whole is limited and it fails to address the types and timing of stressors and their subsequent stress response with precision. Most studies clearly separate emotional/psychological stress (ex. social isolation, rejection, etc.) from physical stress (ex. intense exercise), but fail to recognize the combination of both. For example, a physical challenge may not just be physically stressful, but also emotionally stressful from an anticipatory perspective. Studies that examine only the acute stressor by measuring pre and post-hormonal levels are unable to address pre-acute levels which likely have been affected by anticipatory stress. Some of the contradictory evidence within this field may be influenced by confounding forms of stress that are not controlled for. Furthermore, when trying to identify the relationship between cortisol and progesterone within the stress response, there was a lack of research addressing the possibility that the timing mechanism of each hormone was impacted differently. Most studies examined salivary samples 10-30 minutes post stressful event because it was predetermined that the greatest cortisol response was seen at this time, but this may not be true for the measurement of the progesterone peak in response to the same stressor. More specifically, the study by Kirschbaum et al. (1995) stated that a weakness of their study was that the results were skewed by an outlier and therefore these assumptions likely were made based on skewed data.

Finally, despite the strong fundamental psychology and physiology research completed in the field of stress, limited attention has been paid to the general increase, often unaccounted for

in societal stress. However, throughout the 1980s and 1990s a continual flow of media stories identified more susceptible groups and new varieties of stress, such as “burnout” (Kirby, 2017). The most recent stress outcomes have been identified by a group at the University of Calgary where they found that stress can be contagious (Sterley, 2018). More specifically, the role of women has drastically changed over the last few generations with increased opportunities leading to very different lifestyles with exposure to novel types of stress in the 21st century. Newer longitudinal research should be completed to keep up with these changing lifestyles. Although this ‘state of stress’ is not explicitly addressed in published literature, it is an important consideration that is often highlighted in the grey literature (Global, 2018).

2.9 Summary Gaps in the Current Literature

Stress is a broad and diverse topic with fundamental research dating back to the 1930s extending to modern physiological and population specific research. Due to the nature of measuring stressors, there is both a multitude of varying measures within the literature and a wide number of gaps that need to be addressed. The primary gap in this literature review is the lack of clarity in research examining the relationship between cortisol and progesterone within the stress response. Inconsistency in the literature demonstrates an unclear picture of whether or not cortisol is a stable hormone throughout the MC. Given its wide use as a determinant of stress, this still needs to be determined. While some studies suggest a linear relationship between cortisol and progesterone (increased progesterone = increased cortisol) other studies suggest no relationship. Some studies also suggest it is not the baseline unbound cortisol levels with the linear relationship to progesterone, but the magnitude of the cortisol change in response to stress (increased baseline progesterone = increased Δcortisol).

Secondarily, a clear gap lies within the research involving oral contraceptive methods and the stress response. Many studies commonly use salivary samples rather than blood samples to measure cortisol, progesterone and estrogen hormones. However, Kirschbaum et al. (1995) states the literature suggests that while blood cortisol is increased in OC users, salivary cortisol is decreased in the same population. If this is in fact the case, perhaps the two types of measures cannot be used for both OC users and non-users as results may not be comparable.

Stress and mental health have recently become a priority research topic in universities, specific to the student population. However, most research to date in this population has investigated short-term emotional responses and consequences of stress, rather than the long-term physiological implications. Furthermore, stress is situationally-dependent and more research within different settings and geographical locations needs to be completed as findings of a single study will not be generalizable to all universities.

Stress has a broad scope within health research and because of this there are many large, confusing gaps and weaknesses in the existing literature. Although it is not possible to address all these within a single study, many were taken into consideration when designing the research and study protocol of the CHESS study.

Chapter 3 Methods

3.1 Sample Selection, Recruitment and Ethics

3.1.1 Participant Criteria

Full-time (as defined by the U of C criteria) female undergraduate students at the University of Calgary between the ages 18-28 were the target sample population for this study. Menstrual cycles (MC) may be naturally regular or regulated by a form of birth control including oral contraception (the birth control pill), inter-uterine device (IUDs) or Nuvo Ring. Participants self-reported their MC as regular and between 25-31 days in length (Herrera et al., 2016) and those taking birth control must have used their current form for a minimum of three months prior to the study start. Participants were excluded (n=1) if they were: smokers, pregnant or in the post-partum period, had a diagnosed anxiety disorder, an uncontrolled thyroid condition, and/or were on medication such as corticosteroids, psychoactive drugs or beta-blockers. Also, participants had to have access to a smartphone (iPhone (n=17) or Android (n=2)) to complete the required daily HRV4Training measures. Participants self-reported they were recreationally active, completing at least 30 minutes of moderate intensity physical activity per day (i.e., walking, jogging, cycling, etc.).

3.1.2 Sample Size

Based on results extrapolated from Herrera et al. (2016) measuring salivary progesterone levels post-exposure to acute stress (a protocol resembling the proposed study), an estimated sample size was calculated. Alpha was set to 0.05, with 80% power. The mean change of progesterone levels from pre- to post- acute stress is 4pg/mL with a standard deviation of 5pg/mL. Based on the sample size calculation formula for two groups, the sample size was

determined to be 25. Given an estimated 40% drop-out rate, the sample size needed for this study is 35 participants (see Appendix A).

3.1.3 Ethics

The study was approved by the Conjoint Health Research Ethics Board of Calgary or CHREB (REB 18-0459) on August 28th, 2018. All student-researcher personnel associated with the study completed Tri-council Policy Statement online training prior to beginning data collection (Appendix B). As per ethical requirements, each participant was informed of: the commitments of the study, the data collection procedures, their right to privacy, their right to withdraw from the study at any time and they were given the opportunity to ask any questions prior to commencing the CHESS study. In keeping with these rights, all participants were assigned a participant identification code i.e. NH1 (non-exogenous hormone user one), which was used, rather than names, on all data spreadsheets. All personal information remained under lock and key within the Human Performance Lab and data was saved to Doyle-Baker Lab computers, rather than LC's personal computer. All participants were over the age of 18, and thus, under no circumstance did a parent/guardian need to be present.

3.1.4 Recruitment

The study used a rolling recruitment, beginning after ethics approval and continued throughout the duration of the 2018-19 academic year. Multiple techniques were employed for recruitment while upholding ethics requirements. Posters advertising inclusion/exclusion criteria and contact information for the study (see Appendix C) were placed throughout the University of Calgary's main campus on public bulletin boards including: MacEwan Student Center, Science Theatres, the Faculty of Kinesiology and the Active Living recreation areas. The U of C Faculty

of Kinesiology also posted a write-up regarding the CHESS study which was located on their website and distributed via faculty social media on September 18th, 2018. The posting was further shared by Doyle-Baker lab social media as well as LC's personal social media. The U of C also published a UToday story regarding the CHESS study on October 17th, 2018 which provided inclusion/exclusion criteria and contact information for those readers interested in participating. (Appendix D). Personal recruitment by word-of-mouth and face-to-face explanation was also employed. Finally, four university athletic teams/clubs, namely: rowing, squash, track and field and field hockey, were given information about the study, which was passed along via strength and conditioning personnel or coaches to female athletes.

3.2 Data Collection

3.2.1 Intake Meeting

The "Intake Meeting" was the first step for participants of the CHESS study. Potential participants were invited to the Human Performance Lab in the KNB building. Participants unfamiliar with the HPL were given clear instructions of where to meet. For ethical purposes, and to inform the participants of what the study entailed, participants were given a brief, but inclusive, overview of all aspects and data collection required for the CHESS study, an estimated duration of their involvement (from current date until mid-April, 2019) and what information would be provided to them upon study completion. Commitments included:

1) reporting the first day of menstruation, each cycle throughout the 2018-19 academic year,

2) completing daily HRV measures via the HRV4Training App,

3) tracking their 24-hour dietary intake on days 7 and 21 of each menstrual cycle via the eaTracker application,

4) providing four salivary samples on day 21 of each menstrual cycle (30 minutes after waking, before lunch, before dinner and before bed) and keeping these samples on ice until returned to the HPL,

5) completing a stress questionnaire (SSI) on day 21 of each cycle, and

6) completing a DXA scan on day 21 of their first and last cycle within the CHESS study.

Participants were informed that there was no cost associated with the CHESS study, but there was the minimal risk associated with radiation exposure from the two DXA scans. Participants were also informed that they may withdraw from the study at any time, with or without providing a reason, and could request that the data collected up until that point not be included in the results, if they so desired. They were informed that all information would be kept confidential and under lock and key within the HPL and that data collection would be completed by research coordinator (LC) and assistants (RS, DG) and specific data would be disclosed to committee members Drs. Doyle-Baker, Lebrun (MD) and Murias. Participants were informed that if there were any concerns or outliers within their data, advice would be solicited by LC from either Dr. Doyle-Baker or Dr. Lebrun.

Participants were given the opportunity to ask questions regarding the study procedures and then provided with the consent form (see Appendix E) to read, ask questions once again, and sign. The form was also signed and witnessed by research personnel.

Upon completion of these ethical procedures, participants were given access to the HRV4Training coach application (https://www.hrv4training.com) via a pre-purchased code and

connected with the Doyle-Baker lab's account by a research assistant. Participants were also told to download the eaTracker application (https://www.eatracker.ca) which was free of charge to all account holders with the App Store or Play Store. Finally, participants were asked to complete a baseline SSI questionnaire (Gadzella, 1994). Based on previous validation of this survey at the U of C in Fall 2018 (see Appendix F), any baseline surveys with total scores exceeding 195/255 (Crack, Stokes, & Doyle-Baker, 2018) were excluded from the study (n = 0) and students were guided towards accessible student resources available to help manage stress/anxiety.

Before leaving the HPL, participants were provided with their first salivary sample kit and given instructions to keep the kit in a dry, room temperature environment. Participants were instructed to email the RC on the first day of their period (each cycle) and a schedule for data collection would subsequently be individually provided on a cycle-by-cycle basis.

3.2.2 Personal Characteristics

During the initial intake meeting participants were also asked to self-report: age, date of birth, height, weight, year of undergraduate study, type of birth control (if applicable), if their menstrual cycle is regular (25-31 days), whether or not they are recreationally active (30 minutes of exercise per day minimum), type of smartphone (for the HRV4Training application) and email address for contact. Participants were then grouped into the non-hormone (no birth control or copper IUDs) and exogenous hormone groups (oral contraceptives, hormonal IUDs) based on method of birth control (see Appendix G for a complete list of birth control methods used by participants). Most participants (94%) chose to verbally disclose their university program of study. Participants' university education encompassed a wide range including, but not limited to:

Kinesiology (n=11), Biochemistry (n=1), Health Science (n=2), International Studies (n=2), Environmental Science (n=1) and Geography (n=1).

3.2.3 Approximate Schedule of Data Collection

- September – October 2018: Intake meeting and baseline SSI
- October – November 2018: First Cycle Data Collection

  Daily: HRV

  Day 7: 24-hour dietary intake

  Day 21: 1) 24-hour dietary intake, 2) SSI Questionnaire, 3) Salivary Samples, and 4) In-lab DXA scan
- November 2018 – February 2019: Cycles 2-4 Data Collection

  Daily: HRV

  Day 7: 24-hour dietary intake

  Day 21: 1) 24-hour dietary intake, 2) SSI Questionnaire, and 3) Salivary Samples

  *Christmas break spanned approximately 2-3 weeks in late December and early January, but was not considered a sufficient wash-out period in the study protocol.
- March – April 2019: Final Cycle Data Collection

  Daily: HRV

  Day 7: 24-hour dietary intake

  Day 21: 1) 24-hour dietary intake, 2) SSI Questionnaire, 3) Salivary Samples, 4) In-lab DXA scan, and 5) Godin Leisure-Time Exercise Questionnaire (LTEQ)

3.3 Components of Data Collection

3.3.1 Heart Rate Variability

Participants were instructed on how to use the HRV4Training app so that they could measure their heart rate variability (HRV) every morning upon waking while lying in a supine position breathing normally. The application itself detects whether or not data is useful and if not, the application instructed the participant to try again. All data collected was then automatically transferred to the Doyle-Baker Lab coaching panel account. Participants also used the app to indicate "tags," along with the HRV measures which were self-reported indicators of activity level and health. After approximately one week of measurements, the app provided a baseline range for each participant and when further data was collected, alerted the participant if their HRV normal range changed. The app also provided suggestions for daily activity and rest. Participants were under no study obligation to adhere to the advice given by the app. For the purpose of this thesis, data obtained via the HRV4Training application within the CHESS study will not be used. It will be analyzed in future publications by the Doyle-Baker lab.

3.3.2 Student-Life Stress Inventory

The SSI questionnaire was administered to all participants in person or via email on day 21 of each cycle (in addition to the baseline measure taken at the previously mentioned intake meeting). The questionnaire is composed of 51 stress items in nine different categories: frustrations, conflicts, pressures, changes, self-imposed stress, physiological experiences, emotions, behaviours and cognitive appraisal to determine an overall stress level score (Gadzella, 1994). Participants were instructed to fill-out the questionnaire with regards to their experiences over the previous seven days.

SSI scores were grouped based on the survey validation study completed at the U of C (Crack et al., 2018). One hundred female undergraduate students ranging between 18-26 years of age (mean, SD (±); 20.28 ± 1.78) were surveyed in the validation study to produce a normal distribution of student U of C SSI scores. The mean stress score in this sample was 138.15 ± 28.81 points. A previously employed strategy by Pozos-Radillo et al. (2014), was used for grouping stress scores; SSI scores two standard deviations or more below the sample mean were considered low stress scores, scores two standard deviations or more above the sample mean were high stress scores, and the range between was considered the moderate stress (2014). In this sample, a score of 80 or lower was considered low, 81-194 moderate and 195 or greater was identified as high stress. Furthermore, an individual change in at least 29 points (one standard deviation) was considered a significant change between two SSIs administered to the same participant.

3.3.3 Salivary Sample Collection

Participants were provided with a salivary collection kit at their intake meetings. The kits were from Labrix (https://www.labrix.com) and contained individual shipment packaging which was removed prior to distribution to participants. At the time of distribution, the kits included: the instruction manual, a Styrofoam cooler, a gel-based ice pack, a plastic bag containing four coloured collection tubes and an information form. Participants were instructed to place the ice pack in their freezer the day before collection (i.e., day 20 of their menstrual cycle). They were instructed to fill out the personal information sheet (name, date and date of birth) on the plastic bag containing the samples. They were instructed to read and follow the instructions, but also provided with a brief explanation: "*please take four saliva samples evenly distributed throughout the day: one 30-minutes after waking and before brushing your teeth, one before eating lunch,*

one before eating dinner and one before going to sleep. For each sample, please use the hardware provided in the kit to passively drool into the tube. Please immediately place the samples in the cooler with the ice pack. At the end of the day, please place the bag of samples in your freezer in a clean/dry location. Please bring the samples into the HPL, in the kit with a frozen ice pack, the following business day." All saliva collection and handling of the samples by the participants was unsupervised. When the samples were received by the RC, they were immediately stored in freezer space within the HPL at a standard freezer temperature between -20 to -30 degrees Celsius. Samples were stored in the HPL freezer until they were sent to Labrix and this occurred in two batches (the first batch on January 22, 2019 and the second on April 16, 2019) for assay testing. Labrix procedures and information for the testing of each of the three hormones can be found in a document provided to them by Salimetrics (see Appendix H). In total 59 salivary kits were submitted for analysis and six tubes were not usable in the Labrix analysis. Three tubes had traces of blood and three tubes had leakage during transport. As a result, these profiles were created with one less sample than the others. The morning sample was used to produce a Cortisol Awakening Response (CAR) measure, while the remaining three produced the diurnal curve. For estrogen and progesterone, measures were taken by combining a small sample of each of the four saliva collection tubes for a given day to calculate a daily estrogen value.

3.3.4 DXA Scanning

Dual-energy X-ray absorptiometry (DXA) scanning was completed on day 21 of the first and last menstrual cycle of each participant during the data collection period. The DXA (Hologic QDR 4500, Hologic, Inc., Bedford, MA.) is recognized as the reference method to measure bone mineral density (BMD) and has been previously validated (El Maghraoui & Roux, 2008). It also

provides lean, fat and total body mass measures with accuracy. All participants were instructed to come to the HPL on the two occasions to complete the DXA scan. Before the participant arrived, the DXA machine was calibrated using the spine phantom (daily QC) and the large step phantom calibration tools. The scanner was disinfected. The participant was instructed to wear shorts and a sports bra or tank top with no metal clasps or buttons and to remove all possible metal jewelry prior to entering the scan (see Appendix J for detailed study notes). A computer profile was made for each participant and body weight, height, ethnicity and date of birth were manually entered into the program. The participants were instructed to lie on their backs with arms at their sides and legs in a pigeon toe position while the scan was taken. Scans were analyzed using the computer program after the participant had left.

3.3.5 24-Hour Dietary Intake

The eaTracker application, available free from the registered dieticians of Canada, was used to input dietary intake on days 7 and 21 of each cycle. Participants were instructed to use the food database of the application to input their 24-hour food consumption. For the purpose of this thesis, the data obtained via the eaTracker within the CHESS study will not be used. This data was used in a secondary analysis project within the Doyle-Baker lab.

3.3.6 Godin Leisure-Time Exercise Questionnaire

The Godin Leisure-Time Exercise Questionnaire (LTEQ) was measured once on the last cycle day 21 for each participant (see Appendix I). Physical activity scores were calculated for each participant based on the answers to the questionnaire and its standard calculation formula (Godin, 2011). This questionnaire has been previously validated to categorize individuals into active and insufficiently active groups (Amireault & Godin, 2015).

3.4 Statistical Analysis

Data was organized into Microsoft Excel for Mac and descriptive statistics for all numerical participant characteristics were tabulated as means, standard deviation (±) and/or standard errors (SE) where necessary. IBM SPSS version 22 for Mac was used to perform all other statistical analysis. T-tests were run to analyze possible differences in age, year of study, baseline SSI scores, and activity levels between the two groups. A series of generalized linear models (GLM) were performed to determine if there was a group and/or time effect with: SSI scores, the cortisol awakening response (CAR), noon cortisol levels, evening cortisol levels, night cortisol levels, progesterone and estrogen levels, as well as the estrogen:progesterone (E:P) ratio. The model used was designed to accommodate missing data within the dataset. A regression was used to determine the effect of the slope of the diurnal cortisol curve over the academic year.

3.5 Methods of Knowledge Translation

Given the applicability of the CHESS study to the University of Calgary, research findings will be discussed with Debbie Bruckner, director of the U of C Student Union Wellness Centre upon study completion. To date, the CHESS study has been presented via posters and oral presentations at Perspectives in Exercise, Health and Fitness Conference (Kananaskis, Alberta; October 2018), West Island College (Calgary, Alberta; Feb 2019), Kinesiology Seminars (Calgary, Alberta; March 2019), the 3-minute Thesis Finals (Calgary, Alberta; April 2019), Bodies of Knowledge: Translation in Motion Conference (Toronto, Ontario; May 2019), Exercise in Medicine Conference (Calgary, Alberta; June 2019) and the European College of Sports Sciences Annual Conference (Prague, Czech Republic; July 2019).

Chapter 4 Results

4.1 Participants

Nineteen participants were recruited for data collection in the CHESS study with a mean age of 21.4 ± 2.0 years (range: 20-26 years) and an average year of undergraduate study of 3.6 ± 1.0 (range: 2-6). The participants were separated into two groups (see Tables 4.1 and 4.2): non-exogenous hormone (NH) users or naturally cycling females, and exogenous hormone (EH) users or hormonal birth control users. The groups displayed no difference in height (p=0.19), weight (p=0.17), year of study (p=0.50), baseline stress levels via the SSI or physical activity levels as measured by the LTEQ (p=0.89), but exogenous hormone users were slightly older than non-hormone users (p=0.01). Of the 19 participants, four did not complete the full academic year of data collection. Three drop-outs (NH=2 and EH=1) were due to participant burden and one (NH) was due to commencing an oral contraceptive. More information about drop-outs can be found in data collection notes (Appendix J). These participants did not significantly vary from the CHESS participants as a whole in any of the characteristics listed below.

Table 4.1. Characteristics of the Non-Exogenous Hormone Group (N=9)

	Mean, SD	**Range**
Age (years)	20.2 ± 3.4	19.0-21.0
Height (cm)	164.2 ± 7.1	154.0-173.5
Weight (kg)	64.3 ± 7.1	54.0-81.4
Year of study	3.4 ± 0.7	2.0-4.0
Baseline SSI score	145.0 ± 16.4	123.0-179.0

LTEQ score	65.3 ± 23.6	43.0-110.0

Table 4.2. Characteristics of the Exogenous hormone group (N=10)

	Mean, SD	Range
Age (years)	22.5 ± 2.2	20.0-26.0
Height (cm)	169.0 ± 7.7	158.0-183.0
Weight (kg)	70.7 ± 9.6	50.0-82.4
Year of study	3.7 ± 1.2	2.0-6.0
Baseline SSI score	141 ± 23.9	110.0-174.0
LTEQ score	66.8 ± 18.2	34.0-96.0

4.2 DXA Results

Each participant's first day 21 of data collection began with a DXA scan (one participant opted out for personal reasons; n=18). This analyzed body fat, lean mass and bone mineral density (see Tables 4.3 and 4.4). There was no significant difference in body fat (p=0.5), lean muscle mass (p=0.3) or Z-Score of BMD (p=0.83) between the NH and EH groups. Thirteen participants (72%) completed all months of data collection, including a follow-up DXA on the last, monitored day 21. No significant changes occurred within groups or across groups in body fat (p=0.8), lean muscle mass (p=0.6) or Z-Score BMD (p=0.7) over the duration of CHESS study data collection.

Table 4.3. Baseline DXA Results of the Non-Exogenous Hormone Group (N=8)

	Mean	Range
Body fat (%)	26.9 ± 6.3	18.9-35
Lean muscle mass (g)	44634.0 ± 7459.0	34597.6-57972.3
Z-score BMD	0.66 ± 0.65	-0.5-1.8

Table 4.4. Baseline DXA Results of the Exogenous Hormone Group (N=10)

	Mean	Range
Body fat (%)	28.7 ± 5.1	17.6-34.4
Lean muscle mass (g)	47687.0 ± 5514.0	39104.4-58719.9
Z-score BMD	0.59 ± 0.78	-0.8-2.2

4.3 Statistical Models

A generalized linear model (GLM) was used for the data analysis due to the correlated and unbalanced nature of the data, the generalized estimating equation (GEE) was used within the SPSS GENLIN procedure. This allows estimation for missing data, as long as the data is missing in a random fashion (Ibrahim, 2009; Ibrahim, Chen, Lipsitz, & Herring, 2005). Different amounts of data for each participant were obtained because of the rolling recruitment and missing data. Each GLM was assessed by group (the NH and EH groups), by time (using all participants as one group) and for a time by group interaction (demonstrating whether the group varies with time or the time varies by group). A Wald Chi-Square test was then used to test the simple effects of time within group.

4.4 Student-Life Stress Inventory Results

The Student-Life Stress Inventory (SSI) questionnaire was completed by each participant (n=19) at baseline and once per each subsequent cycle up to a maximum of six times over the academic year. While there was no significant difference in stress levels between the NH and EH groups based on the GEE results of the GLM (see Table 4.5), there was a significant time effect on the questionnaire results.

Table 4.5. Student-Life Stress Inventory Results Over the Academic Year (N=19)

Time	**Group**	**Mean, SE**
Baseline	NH (n=9)	146.4 ± 6.4
	EH (n=10)	142.3 ± 6.8
October	NH (n=2)	127.8 ± 6.7
	EH (n=1)	125.3 ± 5.6
November	NH (n=3)	131.2 ± 9.6
	EH (n=5)	133.3 ± 5.7
December	NH (n=3)	126.1 ± 10.2
	EH (n=5)	140.1 ± 7.5
January	NH (n=5)	128.7 ± 13.0
	EH (n=7)	144.2 ± 10.1
February	NH (n=5)	142.1 ± 9.9
	EH (n=8)	131.9 ± 11.7

March	NH (n=6)	152.2 ± 8.3
	EH (n=8)	137.9 ± 9.4

Significant differences (p<0.05) were observed when comparing the baseline scores to October, November and December and when comparing March scores to October, November and February. Based on the values presented in Table 4.5 and the results of the GLM, stress levels were significantly higher at the beginning (baseline) and end (March) of the academic year than in the middle (see Figure 4.1 below).

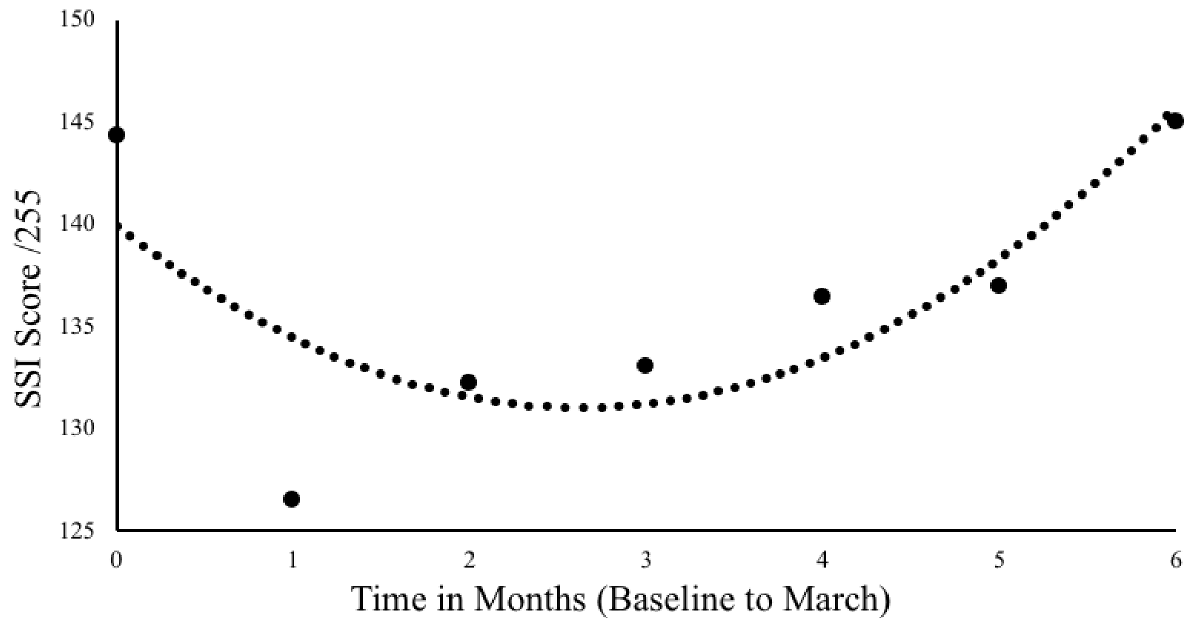

Figure 4-1. SSI scores over the academic year (N=19)

4.5 Cortisol Awakening Response

The cortisol awakening response (CAR) values across each semester for the NH and EH groups are plotted in Figure 4.2. There is a negative slope in the NH group over time throughout each of the two semesters versus the slightly positive trend in the EH group over the same time periods.

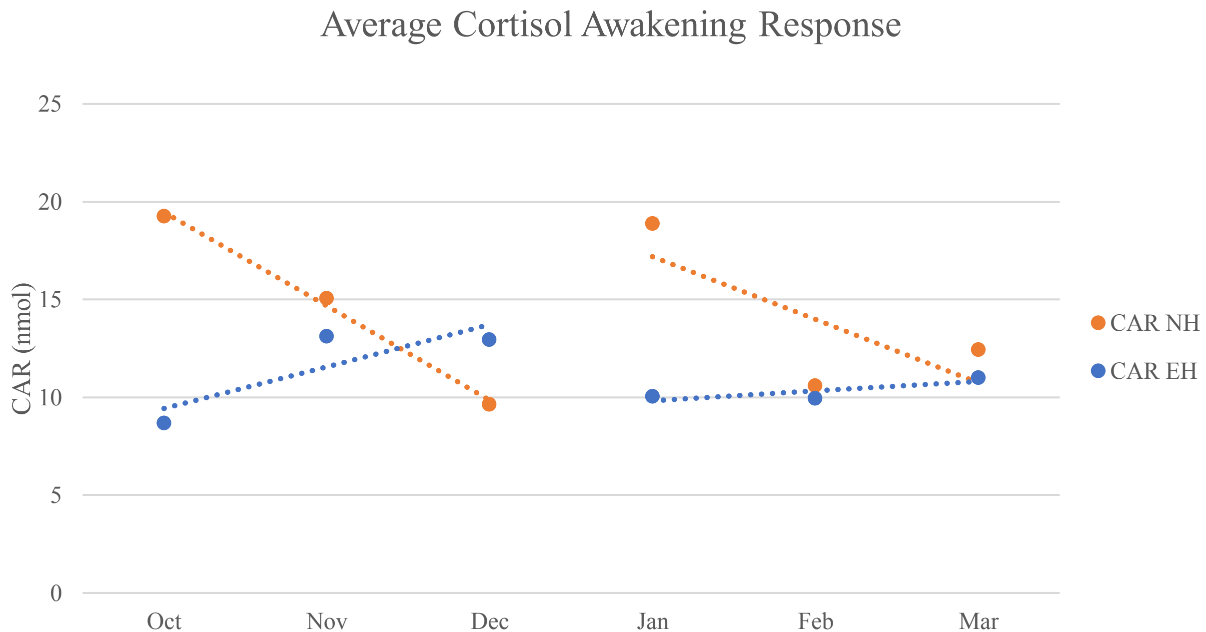

Figure 4-2. Cortisol Awakening Response of NH and EH groups across the academic year (N=19)

The CAR for all participants (n=19) was also analyzed using GLM. A significant time by group effect was detected with Wald Chi-Square test results of $p<0.001$. However, due to the low sample size in month one (October), this time point was removed and the GLM was repeated. No significant time by group interaction was found after this data was removed. Furthermore, there was no significant time effect of all participants without separation of group ($p=0.101$) when October was removed. These results suggest that although the interesting conflicting trends

appear across the two groups, further work with larger sample size and statistical power is needed to attempt to detect significant differences of CAR over time. For all subsequent GLMs conducted, the month of October was thus removed.

4.6 Diurnal Cortisol

4.6.1 Cortisol at Noon

The diurnal cortisol pattern is measured using the noon, evening and nighttime salivary samples. The noon cortisol data was evaluated using GLM and a significant time by group effect (p=0.012) was observed. The Wald Chi-Square test suggests a significant effect of time within the NH group (p=0.001) due to noteworthy differences (p<0.05) between November - February, November - March and January - March. The EH group displays a trend towards significance in the time by group effect (p=0.072) with significant differences (p<0.05) found only between November - December and December - March. When analyzing for a time effect of all participants (n=19) as one group, there was no significant effect; however, results of the Wald Chi-Square trended towards significance (p=0.068). Thus, although significant effects were demonstrated by the population within groups over time, there was no clear picture regarding the impact of academic schedule on noon cortisol specifically and these significant changes are likely produced from different acute stressors.

4.6.2 Cortisol in the Evening

The evening cortisol GLM produced a significant time by group effect with the Wald Chi-Square test giving p=0.005 for the NH group and p=0.044 for the EH group. There was no effect of group alone (p=0.161), but there was a significant effect of time for all participants (p=0.023) based on the pairwise comparison of the estimated marginal means. Similar to the

noon cortisol analysis, there does not appear to be a strong relationship with academic schedule despite detection of significant changes over time.

4.6.3 Nighttime Cortisol

For the nighttime cortisol response, there were no significant effects of group (p=0.843), time (p=0.517) or time by group (p=0.738) found, meaning that nighttime cortisol samples in both groups remained fairly constant throughout the duration of the academic year. Results for all four cortisol responses over time are found in Figures 4.3 and 4.4 (with GLM results in Appendix K).

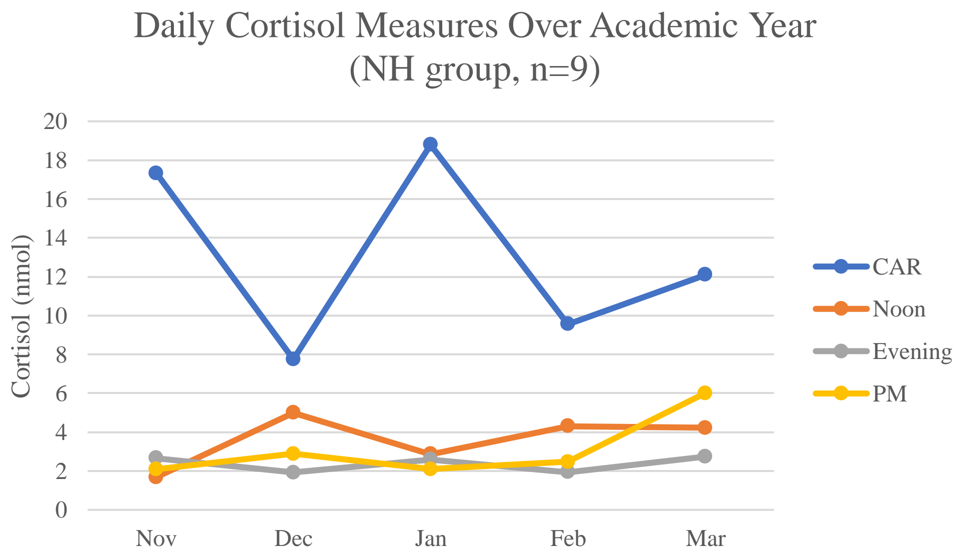

Figure 4-3. Daily cortisol measures over the academic year for the NH group (N=9)

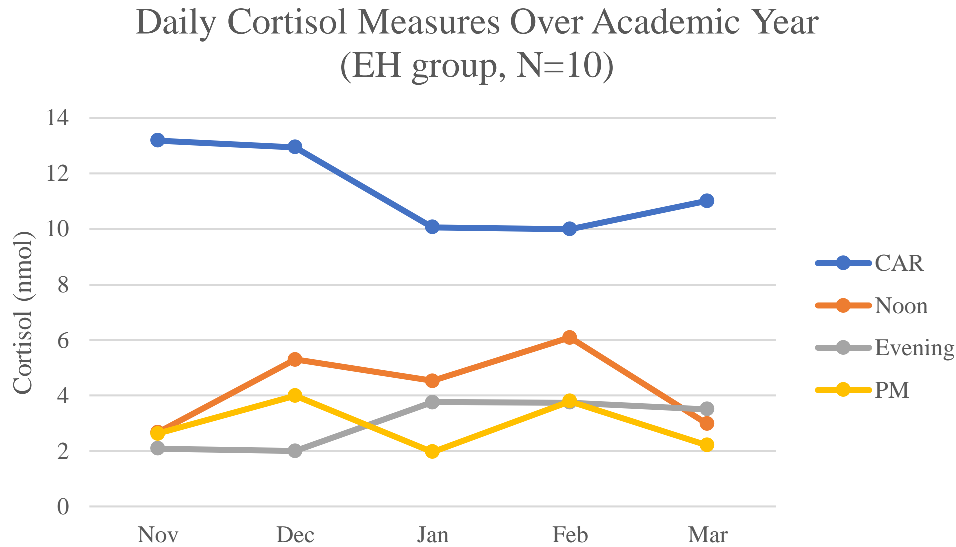

Figure 4-4. Daily cortisol measures over the academic year for the NH group (N=10)

4.6.4 Slope of the Diurnal Cortisol Response

After statistically evaluating each of the three diurnal measures (noon, evening and nighttime) independently, a regression was used to analyze the daily slope of the three time points over the duration of the academic year. There was no significant change in the slope of the diurnal curve over the academic year (p=0.386) and there was no significant effect of time of day based on the Beta value of the regression (p=0.085), although there was a trend towards significance. This suggests that the normal negative slope associated with the daily diurnal cortisol response was not significantly detected in this sample.

4.7 Estrogen and Progesterone Measures

4.7.1 Estrogen Measures

A GLM was run to analyze estrogen levels and the results showed no time (p=0.483) or group (p=0.586) interaction and no time by group effect (p=0.193) with Wald Chi-Square tests

producing no significant time effect in the NH group ($p=0.117$) or EH group ($p=0.803$). Therefore, no differences in estrogen levels between the two groups occurred even though 10 participants used hormonal birth control. Moreover, timing within the academic schedule does not appear to significantly change estrogen levels in this population.

4.7.2 Progesterone Measures

Progesterone measures were procured and analyzed using the same process as estrogen. The GLM displayed significant group ($p=0.001$) and time ($p=0.000$) effects. There was a significant time effect detected by group with Wald Chi-Square results of the NH group ($p=0.001$) and EH group ($p<0.001$). In the NH group there was a significant difference ($p<0.05$) between analyzed months falling in the earlier half of each academic semester (November and January) when compared to months falling in the latter portion of each of the academic semesters (December, February and March). In the EH group, similar results were seen as progesterone was significantly higher ($p<0.05$) in February and March, towards the end of the academic year, when compared to all other time points. These consistent results suggest that academic schedule was impacting progesterone levels in CHESS participants from both groups.

4.7.3 Estrogen:Progesterone Ratio

For each sampling of estrogen and progesterone, a ratio (E:P) was determined for the clinical purpose of testing for progesterone sufficiency or estrogen dominance. The GLM produced a significant group effect ($p=0.009$) and time effect ($p=0.026$). The Wald Chi-Square test demonstrated a significant group effect over time in December, February and March ($p<0.05$). These results were expected based on the previously observed trends in progesterone

levels and the corresponding lack of change in estrogen. Results from the three GLM analyses of estrogen, progesterone and the E:P ratio are found in Table 4.6.

Table 4.6. Tests of Model Effects Results in Estrogen and Progesterone Measures (N=19)

Hormone	Source	Wald chi-square	Sig.
Estrogen	Group	0.30	0.59
	Time	3.47	0.49
	Time * Group	6.08	0.19
Progesterone	Group	11.83	**0.00**
	Time	20.25	**0.00**
	Time * Group	6.28	0.18
E:P	Group	6.74	**0.01**
	Time	11.04	**0.03**
	Time * Group	3.11	0.54

4.8 Progesterone Cortisol Relationship

4.8.1 Progesterone vs Cortisol Awakening Response

A regression was performed to analyze the relationship between progesterone levels (independent variable) and the cortisol awakening response (dependent variable), resulting in an adjusted R-Squared Value of 0.00. There was no statistically significant relationship between the variables as the Beta p-value read 0.328 suggesting no change of CAR with variation in progesterone levels among this sample population.

4.8.2 Progesterone vs Diurnal Cortisol

A second regression analysis was performed to analyze the relationship between progesterone levels and the slope of daily diurnal cortisol. For this analysis, participants were grouped based on clinical levels of progesterone, thus all samples measuring <127pg/mL (Labrix) of progesterone were placed into a subnormal group, versus samples 127pg/mL + being in a normal progesterone group, regardless of birth control status. There were no supra-normal progesterone samples in the CHESS study exceeding the normal progesterone range, and therefore, no such group was included in the regression analysis. The adjusted R-Squared was 0.012 suggesting no relationship between the two variables. There was no group effect of progesterone (subnormal or normal) on diurnal cortisol levels (p=0.346).

Chapter 5 Discussion

Nineteen female undergraduate student volunteers, attending university of Calgary fulltime took part in this study, giving rise to a multitude of results. Because of the limited and contradictory literature existing in this field, the purpose of this discussion will be to critically analyze the results to date, and to help formulate a clearer picture of what research needs to be completed in the future to build off this exploratory study.

5.1 Participants and Characteristics

Participant characteristics in the CHESS study included: age, height, weight, year of study, baseline perceived stress score, physical activity level and body composition. Participants were divided into two groups based on hormonal contraceptive use and no significant difference were found between the groups for all characteristics with the exception of age. The EH group was found to be significantly older than the NH group. Based on a review of the raw data of participant ages, the EH group had one outlier participant, aged 26, increasing the average age. In a larger sample size, this difference would likely not be significant, meaning there is likely not a significant trend towards older students using hormonal birth control in the larger population. There are no other differences between students using hormonal birth controls and those without.

The DXA scan was performed twice during the CHESS study, once on the first day 21 of data collection and once on the last day 21 of data collection. During the first round of DXA examinations, there were no differences in body fat, lean muscle mass or Z-Score BMD across the two groups, suggesting that hormonal birth control does not have a significant effect on body

composition in this population. Body fat percentages ranged from 17 to 35% within the CHESS sample, as compared to the criteria set by the American Council on Exercise identifying 14-24% as athletic and 25-31% as acceptable, while 32% and above as obese (Exercise, 2019). The CHESS average falls within the acceptable range with some participants exceeding the upper limit of normal, but no participants below the lower threshold. Furthermore, of the 18 participants who initially had a DXA performed, 72% completed all months of data collection and had a second DXA completed at the end of the study. There was no significant change in body fat percentage, lean muscle mass or Z-Score BMD from the beginning of the CHESS study to the end, suggesting that although academic stress is present, the stress exposure is not significantly impacting this sample characteristic body composition. This means that the stress exposure is not severe enough or the study did not span a long enough duration to see change. Because of the rolling recruitment and varying MC lengths, the time gap between the first and last cycle DXA scans was different for each participant, also contributing to these results. Previous research in a similar group of university students and athletes, supports this with limited change in body composition (weight, body fat, or fat free mass) despite the stress of being in a negative energy balance (Doyle-Baker, Mclean, & Fung, 2018). This is contrary to animal model research suggesting that stress plays a significant role in body composition changes (Tamashiro, 2007). Body fat percentage would likely change in response to stress induced hypercortisolism. Furthermore, if stress is significantly impacting progesterone and estrogen fluctuation of the MC leading to anovulation, then BMD may be negatively impacted (Park & Song, 1995), potentially resulting in osteopenia and future risk of osteoporosis (Doyle-Baker et al., 2018). Only one participant in this study reached the osteopenia cut off and their score did not significantly worsen over the duration of the CHESS study. These results suggest that stress induced body

composition changes related to fat and BMD did not manifest themselves in the CHESS study participants within the six-month time duration of data collection. Z-score ranges within the CHESS study are comparable to previous research with a student athlete and control group population at the University of Calgary (Doyle-Baker et al., 2018).

5.2 Perceived Stress Levels

Significantly higher SSI scores are observed in the group as a whole (n=19) at baseline (first day of study participation) and in March when compared to October, November, December, January and February which were significantly lower. These results are inconsistent from previous literature, which has suggested that stress levels in female students peak at midterm season and remain elevated until finals each semester (Doyle-Baker, Verge, McClelland, Fung, 2018). There are a number of possible factors at play impacting the distribution of SSI scores over time. It is possible that baseline stress levels are high due to anticipatory stress. A longitudinal study investigating stress levels in nursing students over the duration of their degree suggested that highest stress levels were noted at the beginning of third year. This was attributed to anticipation of both the upcoming academic year and beginning to approach "real world" practicum placements toward the end of their degree (Edwards, Burnard, Bennett, & Hebden, 2010). Although the CHESS study did not include nursing students, it is likely that many students experience this same anticipatory stress and the average year of study within the CHESS sample population was 3.6 yrs., placing them in a similar time of their degree. Thus, baseline stress levels may have been elevated in CHESS participants due to anticipatory stress. SSI results did not significantly differ ($p>0.05$) from the average baseline SSI score in the preliminary U of C study (n=100) and are therefore, likely representative of the U of C female undergraduate population (Crack et al., 2018).

Social support is also well known for reducing stress levels (Zamani-Alavijeh, Raeesi Dehkordi, & Shahry, 2017) and although the CHESS study was not designed to be a social support intervention study, this type of bias may have occurred. After completing the baseline stress questionnaire, participants were regularly in communication with the RC both via email and in person once in each MC when they came in to the HPL to submit samples and complete the monthly SSI. Although this meeting was not designed to be therapeutic in nature, many participants took this opportunity to "vent" their stresses to the RC. Furthermore, the participants were provided a positive and welcoming lab environment so as to reduce the rate of drop-out. The combination of these factors may have provided sufficient yet unintentional social support to the participants resulting in a reduction in stress levels after the baseline questionnaire.

In terms of the stress levels increasing again in March, this fits more closely with the previous literature (Doyle-Baker, Verge, McClelland, Fung, 2018). It is unclear why this increased occurred in the winter semester midterm and final exam period, but not the fall. It is possible that there is a form of additive stress or burnout present by that point in the academic year. Although this was not accounted for in the CHESS study, addressing a measure of student burnout, versus just stress levels, should be considered for in future research to help clarify this result. A study completed at University of Ottawa suggests that burnout rates are significantly higher in female student athletes compared to males (Dubuc-Charbonneau, Durand-Bush, & Forneris, 2014), suggesting that similar to stress levels, burnout may play a larger role in female students' quality of life compared to male counterparts.

5.3 Cortisol Response

When analyzing daily cortisol levels, high Cortisol Awakening Response (CAR) values and a slightly negative diurnal slope is considered normal (Stone et al., 2001), meaning normal levels of cortisol are high in the morning approximately 30-minutes after waking (Clowt al., 2010) and cortisol gradually decreases throughout the day. Thus, cortisol is commonly measured at four time points and separated into the CAR (first sample) and the diurnal cortisol slope (slope of the line of best fit of the remaining three samples) for analysis and we employed this same analysis technique for the CHESS study

5.3.1 The Cortisol Awakening Response

As discussed in section 4.5, there was no significant time or group effect regarding change in CAR over the academic year. This means, that despite fluctuation of self-reported stress levels, especially in the month of March where a statistically significant rise in stress occurred, there was no corresponding effect on the CAR in either group of participants, contrary to the primary study hypothesis. This is somewhat surprising due to the well-known involvement of cortisol in the stress response; however, it is possible that comparing chronic levels of stress versus an acute cortisol measure is not allowing for proper detection of change.

It is also possible that a burnout effect is more impactful in this student population than chronic stress, as previously mentioned with regards to perceived stress levels. Within the CHESS sample as a whole, 94% of participants displayed at least one cycle with a blunted CAR. A normal CAR level is 14-25nmol of cortisol (Doctors Data Labrix, 2019), and 41 sample sets out of a total of 58 collected displayed less than the normal minimum cut-off of 14 nmol. Previous literature suggests that blunted CAR is associated with burnout (Grossi et al., 2005),

therefore supporting the aforementioned possibility that examining burnout in this student sample population may be more applicable to determinants of health than stress alone. Furthermore, some research suggests that quality of sleep plays a role in CAR, but there is mixed results in females compared to males (Fekedulegn et al., 2018). Future research may be warranted to help clarify this relationship so as to not erroneously attribute blunted CAR to burnout, when it may in fact be a result of poor or lack of sleep quality and quantity. Despite the lack of relationship between SSI results and CAR data, based on the result showing 94% of participants with blunted CAR, it is possible that burnout had influenced a hypocortisolism response, in which case an acute, healthy cortisol response was no longer possible. This provides strong evidence that future research in this specific area is required.

5.3.2 Diurnal Cortisol Response

The three time points, in Section 4.6, of cortisol sample collection (lunch, evening and night) were statistically analyzed independently as a function of group and time of the academic year, followed by the analysis of the slope of the diurnal curve measuring cortisol as a function of time of day. The significant time by group effect found by the GLM models analyzing noon and evening cortisol measures suggest that there was a natural variation of cortisol levels throughout the duration of the CHESS data collection. However, there is no clear relationship between a significant increase in stress correlating with a significant increase in cortisol, thereby failing to support the initial study hypothesis stating: there will be a linear correlation between self-reported stress levels and salivary cortisol levels throughout the academic semester. This lack of association between perceived stress levels may be occurring due to a lack of congruency in measuring tools rather than a lack of association within the stress response. While cortisol measures are extremely sensitive to acute and rapid fluctuations in stress throughout the day, the

SSI was not specific enough to give an accurate perceived measure of these changes. Therefore, an association was not found. Daily, rather than monthly, cortisol measures would have to be taken, accompanied by a more sensitive daily stress questionnaire in order to accurately assess this relationship.

A regression analysis was used to observe the cortisol measures as a rate of change throughout the time of day which is a commonly employed means of analyzing diurnal cortisol (Stone et al., 2001). Typically, a slightly negative slope in cortisol levels should be seen throughout the day as cortisol peaks in the morning and is lowest at night. The regression analysis showed no significant rate of change of cortisol based on time of day. This result suggests that HPA axis dysregulation may be a possible impacting factor in this population as individual differences in diurnal cortisol profiles did exist. In a previous study, 15% of participants did not display the typical diurnal curve (Stone et al., 2001) supporting variable results seen in the CHESS study. This evidence is not sufficient alone to determine HPA axis dysregulation. Because cortisol is very sensitive to acute stress and these students were likely managing several acute stressors daily, their diurnal flow of cortisol should be impacted. This suggests that students are in fact experiencing rapid fluctuation in stress. Therefore, our research is important, but the study protocol needs to be refined to more accurately account for these rapid fluctuations within the analysis of chronic stress. To determine whether these results are due to HPA axis dysregulation or a multitude of acute stressors causing healthy stress responses requires further study. Future analysis of diurnal cortisol levels in this population using daily samples, rather than cyclical MC samples, would be needed to attribute these minute changes to the corresponding cause.

5.4 Estrogen and Progesterone Responses

Ten of the 19 participants used hormonal birth control, yet there was no significant difference in estrogen levels between groups, suggesting that the hormonal birth control methods used were not significantly altering estrogen levels on day 21 of the menstrual cycle compared to natural levels. There was also no time effect over the academic year in either group, suggesting academic stress was not impacting the cycle of estrogen. This outcome aligns with previous research by Herrera et al., (2016). Progesterone samples, however, displayed significant group and time effects. The hormonal birth control users exhibited significantly lower progesterone levels on day 21 of their cycles, which is logically the case in order to complete the purpose of hormonal birth control: suppression of ovulation. In accordance with a significant time effect, the NH group displayed significantly increased progesterone levels in December, February and March, while the EH group displayed a significant increase in progesterone levels in the months of February and March. These results tend to support the previous literature suggesting that progesterone is released in response to stress (Herrera et al., 2016), especially given that the month of March displayed a significant increase in both stress levels and progesterone levels across both groups.

From a clinical significance these findings are paradoxical because according to clinical criteria, all 19 females displayed one or more cycles with progesterone insufficiency. Despite limited research in the field, these results are consistent with another study examining more broad effects of medical student stress (Rizvi, Awaiz, Ghanghro, Jafferi, & Aziz, 2010). While this consistent progesterone insufficiency makes sense in the EH group, it is suggesting potential anovulation in the NH group. These trends mean that the increase in progesterone levels during

the end of the semester stressful period actually caused a higher percentage of the NH participants to reach the minimum clinically normal progesterone level of 127pg/mL. Thus, CHESS results support previous literature suggesting an increase in progesterone in response to stress. However, it remains unclear as to whether or not there are actually elevated progesterone related adverse effects of stress or a long-term clinical concern, considering elevated progesterone levels during times of stress are within a normal clinical range. The more appropriate research question moving forward should be related to participants displaying progesterone insufficiency in the remaining months of the academic year, therefore, a possible luteal phase deficiency. It is possible that luteal phase length in this population is shortened or lengthened despite their self-reported regular MC length. If this is the case, salivary measures on day 21 may not be producing an accurate representation of their peak progesterone values. To improve this measurement, it would be beneficial to track both ovulation and salivary hormones. Tracking ovulation is possible via either a urinary analysis or basal body temperature (BBT) to estimate ovulation (Kokts-Porietis, 2019). Progesterone causes an increase in BBT of about 0.5ºF/0.3ºC to 1.0°F/0.6ºC. A sustained increase in BBT is a sign that ovulation has occurred. Testing for ovulation will allow us to more clearly predict when the peak progesterone will occur, rather than simply estimating day 21, so as to obtain a more accurate time-sensitive progesterone measurement and gain clearer insight into the clinical significance of the impact of the stress related progesterone increase. This mixed method approach to menstrual cycle research has also recently been suggested based on a review by Janse de Jonge (2019), who stated a trifold approach to MC verification in research via: calendar counting of the MC, urinary ovulation analysis and salivary progesterone measurement (Janse de Jonge, Thompson, & Han, 2019). With this method, testing of progesterone levels should occur 7-9 days post ovulation for

an increased chance of measuring the true ovulation peak and a minimum value of 16 nmol/L must be detected to ensure luteal phase verification (Janse de Jonge et al., 2019).

5.5 Progesterone and Cortisol Relationship

One of the primary study objectives was to assess the relationship between cortisol and progesterone within the stress response. Previous literature has suggested both that progesterone and cortisol are released in tandem during the stress response, and that baseline levels of progesterone modulate the magnitude of the change in cortisol when exposed to stress (Herrera, 2016). Results of the linear regressions discussed in section 4.8 suggest that there is no correlation between progesterone levels and the value of CAR. Furthermore, when participants' samples were grouped by clinically low progesterone levels (<127pg/mL) and normal progesterone levels (127+pg/mL), there was no effect on the magnitude of the diurnal cortisol slope. As demonstrated by inconsistent and conflicting existing literature regarding the relationship between cortisol and progesterone, the CHESS study results confirm it is difficult to determine the mechanism by which progesterone plays a role in the stress response.

The mechanisms driving cortisol release during stress is an acute response, which is one of the reasons it is difficult to assess the relationship between chronic self-reported stress levels and acute measures of cortisol. Although the literature and CHESS findings suggest that progesterone levels increase with response to stress in a similar acute fashion to cortisol, the primary regulation of progesterone is long-term over a monthly cycle. This means that while cortisol varies acutely due to its designated involvement in fight or flight response, progesterone likely varies on a more longitudinal basis, making is difficult to detect a relationship between the two in terms of chronic stress. When only measuring daily progesterone levels once per cycle,

we cannot identify if acute changes in progesterone do, in fact, occur in addition to its longitudinal variability. Future research should include more frequent measures of progesterone, so as to not only measure chronic variation of peak progesterone levels between cycles, but also to detect possible short-term changes in progesterone due to fluctuation concurrent to the acute cortisol changes.

5.6 Limitations of the CHESS Study

Earlier sections of the discussion described a disconnect between the chronic measures of perceived stress and the extremely sensitive acute changes in cortisol. The sensitivity of the SSI questionnaire may not be enough to effectively addressed the psychological changes in stress that a sensitive measure of cortisol can detect throughout the day. Therefore, a limitation of our tools was that we attempted to analyze the relationship between one chronic measure and one acute measure, perhaps leading to a lack of congruency between the two, and as a result, an inability to effectively examine the relationship.

Another limitation within the CHESS study included the lack of individualized planning for sample collection timing. We employed the previously used technique of measuring the hormonal samples on day 21 of each menstrual cycle (Osakue et al., 2015). It is possible, that females struggle with reporting their true menstrual cycle length (Small, Manatunga, & Marcus, 2007), particularly as it may change over the academic year as previously reported in our lab group (Doyle-baker et al., 2018). In this way, we failed to address an important factor of this research. If stress is in fact impacting menstrual cycle phase length, we cannot expect that menstrual cycles in the NH group to remain constant over a stressful academic year. In turn, day

21 may be an accurate time of progesterone peak in cycle 1, but not in the subsequent cycles, meaning the CHESS study was not robust enough to answer its research objective.

Similar to the concerns related to the relationship between the acute cortisol measures and the chronic SSI measures, a challenge arises when comparing cortisol and progesterone. Given the nature of acute cortisol change versus cyclical progesterone change, completing only one day of salivary sample collection each cycle was not sufficient to measure chronic effects of cortisol or to thoroughly assess the relationship between progesterone and cortisol.

Finally, statistical power was not met during this study due to a low sample size. The sample size of 25 needed to be reached for a statistical power of 80% and our goal was to recruit 35 undergraduate female participants in anticipation of 40% drop-outs. The recruiting process was not as easy as anticipated due to student perception of both participant burden and lack of compensation. Others have found that some students need a lot of time to think about participating. They need to adjust to the idea, figure out their schedules, test whether they trust the researcher, and find out what their friends think (Ed, Speaking, Humor, Transform, & Classroom, 2015) (see Appendix J). Although, we were flexible and creative in increasing the awareness of the study (see Appendix D) and speaking to the benefits, most students did not want to commit as they likely did not find the personal cost–benefit analysis in their favour (Martin, 2014). Moreover, the rolling recruitment schedule and subsequent drop-outs presented a data collection challenge with each participant reporting on a different number of MCs. This required using a GLM to account for both missing data and unequal data points in the analysis. A larger sample size using a similar start date and more standardized timelines for data collection

would be beneficial. Further challenges in day-to-day data collection throughout the CHESS study including recruitment and drop-out information are outlined within the data collection notes in Appendix J.

5.7 Strengths of the CHESS Study

The review of literature in Chapter 2 gave rise to many existing gaps and inconsistencies related to the female physiological stress response. The complexities of the female menstrual cycle, often ignored in research, may have resulted in erroneous attributing of hormonal fluctuations to stress, which are actually caused by menstrual cycle fluctuation and vice versa. Because all women experience slightly different hormonal fluctuations, symptoms and phase lengths during the MCs, it is difficult to create study protocols to effectively produce generalizable results via measurement of hormones and hormonal relationships. Therefore, the main strength of the CHESS study was creating an exploratory protocol that allowed us to observe findings and put forth more controllable and specific research objectives for future studies within the lab and the greater academic community. The statistical analysis and comparison to existing literature has also informed us on areas that should be included for future research as seen in the next section.

5.8 Future Research

A number of critical findings have come out of this exploratory study. Based on the blunted cortisol response and the inconsistencies of the diurnal cortisol curves in 94% of the participants, it seems that HPA axis dysregulation may be playing a critical role in preventing the maintenance of a healthy stress response in this sample population. Future work could gain a better understanding of the stress response in these females by increasing the frequency of saliva

collection and testing of the named hormones, as well as establishing a stress scale/questionnaire that more accurately measures for acute stress related change, rather than chronic. This would allow a better assessment of the relationship between the physiological and psychological measures. Moreover, based on the trend of stress levels observed and the blunted CAR, further research should encompass the use of a burnout scale. Perhaps by the time female students are reaching approximately their third year of undergraduate programs, it is burnout, rather than stress, which is having a significant impact on mental health reporting as shown in surveys like the National College Health Assessment.

Moving forward with regards to the progesterone response, there are two clear findings that must be explored further: 1) the prevalence of progesterone insufficiency among the sample population, and 2) the significant increase in progesterone at the end of an academic year; a stressful period. Our findings identify that progesterone fluctuations are occurring due to stress in this sample population, but the mechanism by which these changes are occurring is not clear. This is similar to cortisol and, therefore, increasing the frequency of testing will allow future research designs to assess not only chronic changes in progesterone between each menstrual cycle, but also to detect whether or not acute progesterone changes occur at the same rate as cortisol. The addition of urine analysis for ovulation will also help future research establish a more individualized tracking schedule for each participant, since it will become easier to establish a true progesterone peak in the mid-luteal phase of the menstrual cycles, despite possible variation in MC phase lengths. This mixed measurement approach to MC research has been recently highlighted to more effectively measure MC phases in research moving forward (Janse de Jonge et al., 2019).

5.9 Conclusions

The exploratory CHESS study was designed to clarify the lack of consistent research regarding the stress response in eumenorrheic undergraduate female students attending university. The CHESS study aimed to provide a greater understanding of the relationship between stress, cortisol and progesterone throughout the hormonal fluctuations of the menstrual cycle. Our study had inconclusive findings related to the relationship between progesterone and cortisol. However, we observed cortisol and HPA axis dysregulation in 94% of this sample population, as well as progesterone insufficiency in all nineteen participants. A significant change in progesterone with exposure to increased academic stress (as measured by the SSI) was seen. These findings suggest that a more refined protocol should be put into place and further research should be conducted within this female undergraduate student population to determine with both sensitivity and specificity the relationship between acute daily changes in cortisol and progesterone, as well as chronic long-term fluctuations in these hormones over an undergraduate university career. This will help provide more clarity related to the short- and long-term health implications of academic stress.

Historically, the MC has been the focus of myth and misinformation (Romans, Clarkson, Einstein, Petrovic, & Stewart, 2012), leading to a gender bias in study recruitment (Cislak et al., 2018). This persists with an underrepresentation in both health and sport science research because researchers have expressed concern regarding the confounding factors associated with the complexity of fluctuations in each woman's MC (Bruinvels et al., 2016). Looking at the larger picture of the MC in relation to research in exercise and health sciences, the CHESS study

was able to contribute to an increase in female-based research and the evidence that research protocols can be effectively completed despite monthly hormonal fluctuation. Results of stress and cortisol levels within the CHESS study did not significantly vary between NH and EH groups despite differences in progesterone. This suggests that MC phase plays a significant role in the body's physiology but may not confound human physiological research as much as suggested. The CHESS study, along with a recently published review by Janse de Jonge et al. (2019), demonstrates that female research protocols can control for the confounding impact of the MC as long as self-report is used in tandem with urinary and salivary analysis to identify MC phase. These are simple, non-invasive measures that can be carried out in studies moving forward. Although the MC cannot simply be ignored in research, we have seen that proper measures are attainable and, therefore, the MC should no longer be a barrier or used as an excuse to exclude females from studies in the fields of exercise and health physiology.

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Appendix A - **Sample Size Calculation**

$N = (Z_{1\text{-}a}+Z_{1\text{-}B})^2 \bullet \sigma^2 / (\mu_A - \mu_0)^2$
$N = 2n$

$N = (1.96+0.84)^2 \bullet 5^2 / (29.5-25.5)^2$
$N = 12.25$
$N = 12.25x2 = 24.5 \sim 25$

With 40% drop-out rate:

$N = 25 \times 1.40 = 35$

Appendix B - **TCPS 2: Core Certificate**

Appendix C - **Recruitment Poster**

The CHESS Study

Are you a full-time female UG student between 18-28 years of age?

You may be eligible to participate in this investigating hormone response to stress study!

We would like to test your aerobic fitness levels during your menstrual cycle over the 2018-2019 academic year?

If you are not taking any birth control, are recreationally active, and willing to do salivary hormone levels and interested in participating, please contact:

Laura Crack in the Doyle-Baker Lab at the University of Calgary:

Title: Changes in Hormones with Response to Student Stress. PI. Dr. PK. Doyle-Baker.
This study has been approved by the University of Calgary Conjoint Health Ethics Board (REB 18-0459)

Appendix D - **UToday Story**

Kinesiology researcher seeks female students to join study on acute and chronic stress
10 Participants will contribute to data on female hormone response to stress over course of this academic year
By Stacy McGuire, Faculty of Kinesiology

October 17, 2018

Daniela Garcia Orellana, a fourth-year kinesiology student, is helping with the Change in Hormones with Exposure to Student Stress (CHESS) study led by P.K. Doyle-Baker, professor in the Faculty of Kinesiology. ***Photo by Riley Brandt, University of Calgary***

Some claim that university is the happiest time of your life. It may be, but it is still a very stressful time for many students. Dr. P.K. Doyle-Baker, DrPH, professor in the Faculty of Kinesiology, is investigating how stress influences a female student's hormones and eating patterns.

"Stress is a silent thief," says Doyle-Baker. "It can do many things to our bodies over time, even contribute to bone mineral density loss."

Ninety per cent of students expressed feeling overwhelmed at some point in the year, according to a University of Calgary campus-wide student survey on health in 2013.

Over the next six months, Doyle-Baker wants to understand how chronic and acute stress effects female students, an under-represented population in health and sport research.

"Any student can tell you they have stress," says Doyle-Baker. "But we want to know when and how much stress and see what it does to their physiology so we can strategize on reducing any issues early on."

Daniela Garcia Orellana, a fourth-year kinesiology student helping with the study, agrees it is important to find tools to combat stress at university. "Though this has been the best time of my life, university can be stressful if you are trying to get that high GPA for further studies," she says.

She credits a strong support network with other students, taking part in sports, and volunteering for helping her maintain the balance necessary to cope with stress.

Three-part study examines stress and hormones

The study, Change in Hormones with Exposure to Student Stress (CHESS), is fitting, says Doyle-Baker.

"First, we want to understand chronic or long-term stress. This we will measure through saliva," she explains. "We also want to study acute stress, and this will we measure by testing aerobic capacity. The third piece of the study is to examine hormone levels."

Participants will have estrogen and progesterone levels tested through salivary measures to see what changes occur during acute stress.

The final piece of the study includes an eating inventory to understand participants' energy intake. "We want to observe their choice of protein, carbs and fat during their menstrual cycle and see if they are influenced by stress, hormones or both," says Doyle-Baker.

Female participants needed over course of academic year

"Students who engage in research can learn so much about themselves and others. It can enrich their student experience," says Doyle-Baker.

Doyle-Baker is recruiting full-time female students aged 18 to 28 to take part in CHESS over the course of the academic year.

"We need more women to take part in research, and what a great legacy students can provide, to improve the health of future generations of students and women."

Learn more about eligibility, or to take part in the study, contact Laura.crack@ucalgary.ca,

Interested in participating in research at UCalgary? Check out more opportunities.

The research is funded by Sport Science Association of Alberta (SSAA) through Alberta Sport Connection. The survey statistic on stress is taken from the Campus Mental Health Strategy. The statement on under-represented female population in health and sport research is taken from Bruinvels G, et al. (2017). Br J Sports Med., 51:487–488.

Appendix E - **Consent Form**

Consent Form

TITLE: Changes in Hormones with Exposure to Student Stress (CHESS)

INVESTIGATORS: Drs. Patricia K. Doyle-Baker, Dr. PH, (CSEP-CEP); Juan Murias (PhD), Connie lebrun (MD), Laura E. Crack, BSc(Hons), (MSc Candidate)

laura.crack@ucalgary.ca

This consent form is only part of the process of informed consent. It should give you the basic idea of what the research is about and what your participation will involve. If you would like more detail about something mentioned here, or information not included here, please ask. Take the time to read this carefully and to understand any accompanying information. You will receive a copy of this form.

BACKGROUND

There is limited knowledge on how the menstrual cycle is affected by stress in our life. There are only a few studies looking at the hormones of females and how stress changes them. Currently there are no published studies involving progesterone's response to stress in naturally cycling female students, not on birth control attending full-time university.

WHAT IS THE PURPOSE OF THE STUDY?

The purpose of this study is to observe over an academic semester changes in: 1) self-reported stress levels in female undergraduate students, and 2) cortisol and progesterone while exposing female undergraduate students to an exercise stress during the different menstrual cycle phases.

WHAT WOULD I HAVE TO DO?

You must:

- Be in good health based on prescreening tool [Physical Activity Readiness Questionnaire (PAR-Q)] with normal resting heart rate (HR) and blood pressure (BP)
- Have a menstrual cycle not shorter than 25 days or greater than 31 days and not currently on any form of contraceptive (oral or transdermal)
- Be active for a minimum of 30 minutes per day at a recreational level.
- Agree to monitor your cortisol and progesterone levels via salivary sample once per week over 5-6 complete menstrual cycles.
- Agree to complete a Stress Symptom Inventory (SSI) once per month for the duration of the study.
- Agree to complete 3 maximal physical exertion tests, one during each the three menstrual cycles monitored in the intervention phase of the study.

WHAT ARE THE RISKS?

The risks associated with this study may include discomfort and fatigue associated with the maximal physical exertion test and the DXA poses no obvious physical risk above the normal radiation from the sun on a bright day in Calgary.

WILL I BENEFIT IF I TAKE PART?

If you agree to participate, upon completion of the study you will gain access to your oxygen uptake levels at maximal exertion which can be used as one indicator of aerobic fitness level and your baseline hormone levels.

WILL WE BE PAID FOR PARTICIPATING, OR DO WE HAVE TO PAY FOR ANYTHING?

There will be no financial compensation to you, and there will be no costs to participate in this study.

WILL MY RECORDS BE KEPT PRIVATE?

All of the information collected will remain strictly confidential. Only the investigators responsible for this study, the research assistant (RA) who will be doing the baseline assessments, and the University of Calgary Conjoint Health Research Ethics Board will have access to this information. Confidentiality will be protected by using a study identification (ID) number in the database. Any results of the study reported will in no way identify you the study participant.

IF I SUFFER A RESEARCH-RELATED INJURY, WILL I BE COMPENSATED?

In the event that you suffer an injury as a result of participating in this research, no compensation will be provided to you by the University of Calgary or the researchers. You still have all your legal rights. Nothing said in this consent form alters your right to seek damages.

SIGNATURES

Your signature on this form indicates that you have understood to your satisfaction the information regarding your participation in the research project and agree to participate as a participant. In no way does this waive your legal rights nor release the investigators or involved institutions from their legal and professional responsibilities. You are free to withdraw from the study at any time without jeopardizing your health care. If you have further questions concerning matters related to this research, please contact:

Dr. PK Doyle-Baker (Principle Investigator): (403) 220-7034

If you have any questions concerning your rights as a possible participant in this research, please contact the Chair, Conjoint Health Research Ethics Board, University of Calgary at 403-220-7990.

______________________________	______________________________
Participant's Name	Signature and Date
______________________________	______________________________
Investigator/Delegate's Name	Signature and Date
______________________________	______________________________
Witness' Name	Signature and Date

The University of Calgary Conjoint Health Research Ethics Board has approved this research.

A signed copy of this consent form has been given to you to keep for your records and reference.

Appendix F - **SSI Baseline Study Results**

UNIVERSITY OF CALGARY
FACULTY OF KINESIOLOGY

Doyle-Baker Lab

The tables turned: how we became stressed by measuring stress.

Laura E. Crack[1], Rachel E. Stokes[1], Patricia K. Doyle-Baker[1,2]

[1]Faculty of Kinesiology, [2]Faculty of Environmental Design, University of Calgary, Calgary, Alberta.

BACKGROUND

The National College Health Assessment (NCHA) recently reported that a fifth of Canadian postsecondary students are depressed, anxious or battling other mental health issues. Students surveyed in 2016 reported good overall health, but when compared to 2013 it had decreased by 8%.[1]

Stress is considered a risk factor for mental illness among university students and becoming more prevalent due to the large range of academic, social and personal challenges.

A large majority (71%) of U. of Alberta students, self-reported their lives as either stressful or very stressful, while only 16% reported acceptable levels of stress.[2]

Female students are more likely to report unacceptable levels of stress[2] and reach higher levels of stress earlier in the semester. [3]

CONTACT

pdoyleba@ucalgary.ca
laura.crack@ucalgary.ca
http://www.doylebakerlab.com/

INTRODUCTION

We wanted to identify undergraduates students' perceived stress at the start of the fall academic (2018) semester for a larger study called CHESS (Change in hormones effected by student stress; REB 18-0459).

- Pilot tested (n=15) using a previously validated Student-Life Stress Inventory (SSI) (Gazelle et al., 1994).

We were shocked to find out that self-reported scores were above the maximum cut off value of 171, that we cited in ethics from the Gazelle et al. study (1994).

PURPOSE

Our study purpose was to develop an optimal SSI cut-off score (i.e. best estimate of the presence of unhealthy stress) in the same population as our proposed study group, i.e., female undergraduates attending a large university in Alberta.

METHODS

A convenience sample of undergraduate female students were recruited (N=100; including n=15)

- Students were approached in the campus hallways and classrooms during the first 3-weeks of the September (2018)

A cross-sectional study design

- Stress level was measured only, once.

Survey Questionnaire

- Composed of 51 stress items across 9 categories of stress
  - Frustrations, Conflicts, Pressures, Changes, Self-Imposed Stress, Physiological, Emotional and Behavioural Reactions to stress and Cognitive Appraisal.
    - Each stress item was ranked on a scale of 1-5 (1 being least applicable, to 5 most applicable).
      - Maximum score total = 255 points

Descriptive Statistics

- Frequency distribution grouped into 8-point intervals (see Figure 1).
- Score were tabulated in an excel spreadsheet
  - Means and standard deviations (± SD) were used to establish low, moderate and high levels of stress.

Cut-offs scores

- We employed a common strategy for determining cut-offs scores based on the population mean (see Figure 2).
  - Participants with low stress levels scored 2 or more SD below the sample mean
  - Participants with high stress levels scored 2 or more SD above the sample mean.

RESULTS

Mean

- age 20.28 ± 1.78; range 18-26 years
- SSI score 138.15 ± 28.81 points

Figure 1. Frequency distribution of SSI scores

Percentiles
(See Figure 2 cut-off scores)
Minimum 73; Maximum 206
25th Percentile 117; 50th Percentile 136; 75th Percentile 155

Figure 2. Calculated criteria values for stress level groupings

Each category had a maximum score out of 5

- Average score across the categories of stress:
  - Frustrations: 2.51 ± 1.29
  - Conflicts: 2.89 ± 1.14
  - Pressures: 3.65 ± 1.24
  - Changes: 2.77 ± 1.37
  - Self-Imposed Stress: 3.65 ± 1.24
  - Physiological Stress Response: 2.43 ± 1.45
  - Emotional Stress Response: 2.97 ± 1.50
  - Behavioural Stress Response: 2.14 ± 1.41

DISCUSSION

Based on previous literature, we were able to use descriptive statistics of the sample population to determine both high and low stress level cutoff scores (Pozos-Radillo, 2014) on the SSI (two SD either above or below the mean, respectively).

- We were able to determine that a significant change in stress level occurs when an participant's survey results vary by at least one SD or 28.8 points.

Our results can help classify participants, identify significant change and provide a cutoff value for high stress.

- This cutoff value will be used to guide participant to available campus resources that can help with their stress.

In keeping with recent media reporting and the National College Health Assessment results, the of U of C female undergraduate students sampled displayed that perceived stress levels are concerning, even during the first few weeks of the semester before exams or assignments are in full swing.

- This raises the question: what impact does stress have on physical health during periods of highest academic stress (i.e. exams)?

The CHESS study will be completed using these new baseline stress cut offs. The study at will assess the impact of stress on physical health including: female hormone cycles, heart rate variability/fatigue, dietary intake of macro nutrients and bone mineral density.

- We, as researchers are more comfortable with this stress cutoff score and more mindful that stress occurs in both participant and investigators.

Take Away Messages!

- Short-term coping includes walking/talking for social support.[6]
- *UFlourish* is a UofCalgary program designed with a series of activities aimed at building resiliency and positive mental health as small meaningful changes can be made even when there are time restrictions and financial limitations in a student's life.

We need to remember that 'the absence of a mental illness is not the same as positive mental health'.

REFEERENCES

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7. Photo: Morin, Krystal (April 2016). Flipping the Classroom. www.ubishops.ca/future-current-students/

Appendix G - **Birth Control Methods**

Oral Contraceptives	**Hormonal IUDs**	**Vaginal Rings**
Yaz	Mirena	NuvoRing
Lo Loestrin Fe	Kyleena	

Appendix H - **Labrix Hormone Testing Procedures**

High Sensitivity

SALIVARY 17β-ESTRADIOL

ENZYME IMMUNOASSAY KIT

For Research Use Only
Not for use in Diagnostic Procedures

Item No. 1-3702,
(Single) 96-Well Kit;
1-3702-5, (5-Pack)
480 Wells

101 Innovation Boulevard • Suite 302 • State College, PA 16803
1.800.790.2258 • support@salimetrics.com • salimetrics.com

Intended Use

The Salimetrics® 17β-Estradiol Enzyme Immunoassay Kit is a competitive immunoassay specifically designed and validated for the quantitative measurement of salivary Estradiol. It is not intended for diagnostic use. It is intended only for research use in humans and some animals. Salimetrics has not validated this kit for serum or plasma samples.

Warning: The drug fulvestrant (FASLODEX®) has been shown to cross react with antibodies used in Estradiol immunoassays and may cause falsely elevated Estradiol results. Due to the risk of this cross reactivity, the Salimetrics® High Sensitivity Salivary 17β-Estradiol Enzyme Immunoassay Kit should not be used for individuals being treated with the drug fulvestrant (FASLODEX®). Fulvestrant (FASLODEX®) is used to treat a certain type of estrogen receptor positive breast cancer in postmenopausal women. Falsely elevated Estradiol results could lead to misinterpretation of the menopausal status of these women, resulting in the fulvestrant (FASLODEX®) treatment being incorrectly altered or discontinued.

Please read the complete kit insert before performing this assay. Failure to follow kit procedure and recommendations for saliva collection and sample handling may result in unreliable values.

For further information about this kit, its application, or the procedures in this insert, please contact the technical service team at Salimetrics or your local sales representative.

Introduction

Estradiol (17β-Estradiol, E2, 1,3,5(10)-estratriene-3, 17β-diol), a steroid hormone, is produced primarily by the ovarian follicles from testosterone (1,2). Estradiol is the most active naturally secreted estrogen (1). In men, Estradiol originates in the testes and from extraglandular conversion of androgens (1).

Circulating Estradiol levels are relatively high at birth in both males and females, but decrease postnatally (2). In prepubertal children and men, levels are non-cyclic and low. During puberty, there are gradual increases in Estradiol levels in both males and females.
Interactions between luteinizing hormone (LH) and follicle-stimulating hormone (FSH) cause the release of Estradiol from the ovaries in premenopausal women. Estradiol secretion is low in postmenopausal women.

Research concerning Estradiol has focused predominantly on reproductive issues such as conception, ovulation, infertility, and menopause (3,4). Yet, Estradiol affects a diversity of biological processes involved with reproductive capacity, (5) establishment and maintenance of pregnancy, (6) parenting, (7) coronary artery disease, (8) immunocompetence, (9) cancer susceptibility, (10) and neuroprotection (11). Estradiol is also believed to affect individual differences in cognitive and socioemotional processes as well as psychopathology (12,13).

Estrogens have been measured by many immunoassay methods. Studies suggest that Estradiol can be accurately measured in saliva (3,4,14,15).

Test Principle

This is a competitive immunoassay kit. Estradiol in standards and samples compete with Estradiol conjugated to horseradish peroxidase for the antibody binding sites on a microtitre plate. After incubation, unbound components are washed away. Bound Estradiol Enzyme Conjugate is measured by the reaction of the horseradish peroxidase enzyme to the substrate tetramethylbenzidine (TMB). This reaction produces a blue color. A yellow color is formed after stopping the reaction with an acidic solution. The optical density is read on a standard plate reader at 450 nm. The amount of Estradiol Enzyme Conjugate detected is inversely proportional to the amount of Estradiol present in the sample (16).

Safety Precautions

Read Safety Data Sheets before handling reagents.

Hazardous Ingredients

Liquid Stop Solution is caustic; use with care. We recommend the procedures listed below for all kit reagents.

Handling

Follow good laboratory practices when handling kit reagents. Laboratory coats, gloves, and safety goggles are recommended. Wipe up spills using appropriate absorbent materials while wearing protective clothing. Follow local regulations for disposal.

Emergency Exposure Measures

In case of contact, immediately wash skin or flush eyes with water for 15 minutes. Remove contaminated clothing. If inhaled, remove individual to fresh air. If individual experiences difficulty breathing call a physician.

The above information is believed to be accurate but is not all-inclusive. This information should be used only as a guide. Salimetrics will not be liable for accidents or damage resulting from misuse of product.

Safety Data Sheets are available by contacting Salimetrics at support@salimetrics.com (See www.salimetrics.com for alternative contact options).

General Kit Use Advice

- This kit uses break-apart microtitre strips. You may run less than a full plate. Unused wells must be stored at 2-8°C in the foil pouch with desiccant and used in the frame provided.
- Avoid microbial contamination of opened reagents. Salimetrics recommends using opened reagents within one month. Store all reagents at 2-8°C.
- The quantity of reagent provided with a single kit is sufficient for three partial runs. The volumes of wash buffer and enzyme conjugate prepared for assays using less than a full plate should be scaled down accordingly, keeping the same dilution ratio.
- Do not mix components from different lots of kits.
- To ensure highest quality assay results, pipetting of samples and reagents must be done as quickly as possible (without interruption) across the plate. Ideally, the process should be completed within 20 minutes or less.
- When using a multichannel pipette to add reagents, always follow the same sequence when adding all reagents so that the incubation time is the same for all wells.
- When running multiple plates, or multiple sets of strips, a standard curve must be run with each individual plate and/or set of strips.
- The temperature of the laboratory may affect assays. Salimetrics' kits have been validated at 68-74°F (20-23.3°C). Higher or lower temperatures may affect OD values.
- Routine calibration of pipettes and other equipment is critical for the best possible assay performance.
- When mixing plates during assay procedures, avoid speeds that spill the contents of the wells.

Storage

All unopened components of this kit are stable at 2-8°C until the kit's expiration date.

pH Indicator

Estradiol values from samples with a pH $\leq$ 5.0 or $\geq$ 9.0 may be inaccurate. A pH indicator in the HS Estradiol Assay Diluent alerts the user to samples with high or low pH values. Upon addition of the HS Estradiol Assay Diluent, acidic samples will turn yellow and alkaline samples will turn purple. Dark yellow or purple wells indicate that a pH value for that sample should be obtained using pH strips. Samples with a pH $\leq$ 5.0 or $\geq$ 9.0 should be recollected.

Specimen Collection

Avoid sample collection within 60 minutes after eating a major meal or within 12 hours after consuming alcohol. Acidic or high sugar foods can compromise assay performance by lowering sample pH and influencing bacterial growth. To minimize these factors, rinse mouth thoroughly with water 10 minutes before sample is collected.

Collect whole saliva by unstimulated passive drool. Donors may tilt the head forward, allowing the saliva to pool on the floor of the mouth, then pass the saliva through the SalivaBio Collection Aid (SCA) into a polypropylene vial. Collection protocols/methods are available online at www.salimetrics.com or upon request.

Samples visibly contaminated with blood should be recollected. Samples may be screened for possible blood contamination (17,18) using our Blood Contamination EIA Kit (Item Nos. 1- 1302/1-1302-5). Do not use dipsticks, which result in false positive values due to salivary enzymes.

Record the time and date of specimen collection.

Sample Handling and Preparation

After collection, it is important to keep samples cold in order to avoid bacterial growth in the specimen. Refrigerate sample within 30 minutes, and freeze at or below -20ºC within 4 hours of collection. (Samples may be stored at -20ºC for up to 6 months.) For long term storage, refer to the Salimetrics Collection and Handling Advice Booklet.

Do not add sodiumazide to saliva samples as a preservative, as it may cause interference in the immunoassay.

On day of assay, thaw the saliva samples completely, vortex, and centrifuge at 1500 x g for 15 minutes. Freezing saliva samples will precipitate mucins. Centrifuging removes mucins and other particulate matter which may interfere with antibody binding and affect results. Samples should be at room temperature before adding to assay plate. Pipette clear sample into appropriate wells. Re-freeze saliva samples as soon as possible after adding to the assay plate. Re-centrifuge saliva samples each time that they are thawed. Avoid multiple freeze-thaw cycles.

Materials Supplied with Single Kit

	Item	Quantity/Size
1	**Microtitre Plate** Coated with rabbit anti-Estradiol antibodies.	1/96 well
2	**HS Estradiol Standard** 32 pg/mL, in a saliva-like matrix. Serially dilute before use according to Reagent Preparation. Contains: Estradiol, buffer, preservative.	1 vial / 1.6 mL
3	**HS Estradiol Controls** High, Low, in a saliva-like matrix. Ready to use. Contain: Estradiol, buffer, preservative.	2 vials / 1 mL each
4	**Estradiol Enzyme Conjugate** Concentrate. Dilute before use with HS Estradiol Assay Diluent. (See step 5 of Procedure.) Contains: Estradiol conjugated to HRP, preservative.	1 vial / 50 µL
5	**HS Estradiol Assay Diluent** Contains: phosphate buffer, pH indicator, preservative.	1 bottle / 60 mL
6	**Wash Buffer Concentrate (10X)** Dilute before use according to Reagent Preparation. Contains: phosphate buffer, detergent, preservative.	1 bottle / 100 mL
7	**TMB Substrate Solution** Non-toxic, ready to use.	1 bottle / 25 mL
8	**Stop Solution**	1 bottle / 12.5 mL
9	**Non-Specific Binding (NSB) Wells** Do not contain anti-Estradiol antibody. Break off and insert as blanks (optional) where needed.	1 strip
10	**Adhesive Plate Covers**	2

Materials Needed But Not Supplied

- Precision pipette to deliver 15 µL, 100 µL, and 300 µL
- Precision multichannel pipette to deliver 50 µL, 100 µL, and 200 µL
- Vortex
- Plate rotator with 0.08-0.17 inch orbit capable of 500 rpm
- Plate reader with 450 nm and 620 to 630 nm reference filters
- Computer software for data reduction
- Deionized water
- Reagent reservoirs
- One disposable polypropylene tube to hold at least 12 mL
- Five small disposable polypropylene tubes for dilution of standard
- Pipette tips
- Serological pipette to deliver up to 12 mL
- Centrifuge capable of 1500 x g

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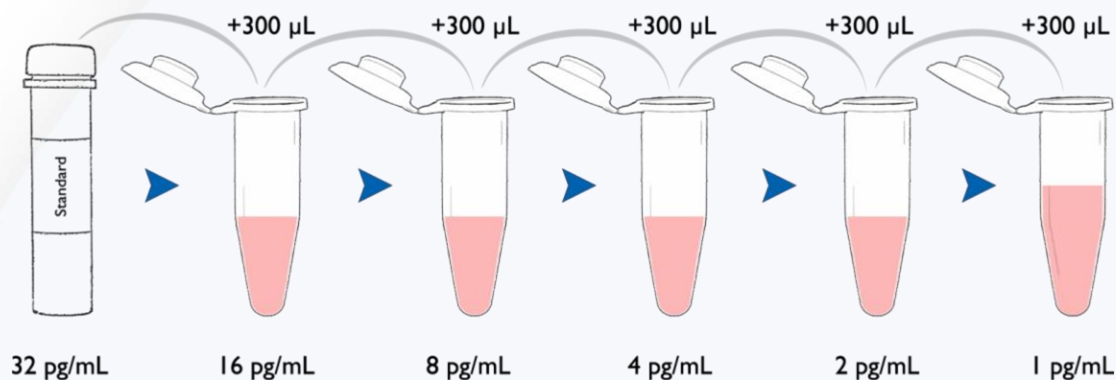
Reagent Preparation

- Bring all reagents to room temperature and mix before use. A minimum of 1.5 hours is recommended for the 12 mL of HS Estradiol Assay Diluent used in Step 5 (conjugate dilution) to come to room temperature.
- Bring Microtitre Plate to room temperature before use. **It is important to keep the foil pouch with the plate strips closed until warmed to room temperature, as humidity may have an effect on the coated wells.**
- Prepare 1X wash buffer by diluting Wash Buffer Concentrate (10X) 10-fold with room-temperature deionized water (100 mL of Wash Buffer Concentrate (10X) to 900 mL of deionized water). **Dilute only enough for current day's use and discard any leftover reagent.** (If precipitate has formed in the concentrated wash buffer, it may be heated to 40°C for 15 minutes. Cool to room temperature before use in assay.)
- Prepare serial dilutions of the HS Estradiol Standard as follows:
    - Label five polypropylene microcentrifuge tubes or other small tubes 2 through 6.
    - Pipette 300 µL of HS Estradiol Assay Diluent into tubes 2 through 6.
    - Serially dilute the standard 2X by adding 300 µL of the 32 pg/mL standard (tube 1) to tube 2. Mix well.
    - After changing pipette tips, remove 300 µL from tube 2 to tube 3. Mix well.
    - Continue for tubes 4, 5, and 6.
    - The final concentrations of standards for tubes 1 through 6 are, respectively, 32 pg/mL, 16 pg/mL, 8 pg/mL, 4 pg/mL, 2 pg/mL, and 1 pg/mL. Standard concentrations in pmol/L are 117, 58.5, 29, 14.6, 7.3, and 3.65 respectively.



Procedure

Step 1: Read and prepare reagents according to the Reagent Preparation section before beginning assay. Determine your plate layout. Here is a suggested layout. (Standards, controls, and saliva samples should be assayed in duplicate.)

	1	2	3	4	5	6	7	8	9	10	11	12
A	32 Std	32 Std	Ctrl-H	Ctrl-H								
B	16 Std	16 Std	Ctrl-L	Ctrl-L								
C	8 Std	8 Std	SMP-1	SMP-1								
D	4 Std	4 Std	SMP-2	SMP-2								
E	2 Std	2 Std	SMP-3	SMP-3								
F	1 Std	1 Std	SMP-4	SMP-4								
G	Zero	Zero	SMP-5	SMP-5								
H	NSB*	NSB*	SMP-6	SMP-6								

*NSB = Non-specific binding wells. These may serve as blanks. Use is optional.

Step 2: Keep the desired number of strips in the strip holder and place the remaining strips back in the foil pouch. If you choose to place non-specific binding wells in H-1, 2, remove strips 1 and 2 from the strip holder and break off the bottom wells. Place the strips back into the strip holder leaving H-1, 2 blank. Break off 2 NSB wells from the strip of NSB wells included in the foil pouch. Place in H-1, 2. Alternatively, NSBs may be placed wherever you choose on the plate. Reseal the foil pouch with unused wells and desiccant. Store at 2-8°C.

Cautions: 1. Extra NSB wells should not be used for determination of standards, controls, or unknowns.
2. Do not insert wells from one plate into a different plate.

Step 3: Pipette 12 mL of HS Estradiol Assay Diluent into the disposable tube. (Scale down proportionally if using less than the entire plate.) Set aside for Step 5.

Step 4:

- Pipette 100 µL of standards, controls, and saliva samples into appropriate wells.
- Pipette 100 µL of HS Estradiol Assay Diluent into 2 wells to serve as the zero.
- Pipette 100 µL of HS Estradiol Assay Diluent into each NSB well.

Step 5: Dilute the Enzyme Conjugate 1:800 by adding 15 µL of the conjugate to the 12 mL tube of HS Estradiol Assay Diluent. (Scale down proportionally if not using the entire plate.) Conjugate tube may be centrifuged for a few minutes to bring the liquid down to the tube bottom. Immediately mix the diluted conjugate solution and add 100 µL to each well using a multichannel pipette.

Step 6: Place adhesive cover provided over plate. Mix plate on a plate rotator for 5 minutes at 500 rpm and incubate at room temperature for a total of 2 hours.

Step 7: Wash the plate 4 times with 1X wash buffer. A plate washer is recommended. However, washing may be done by gently squirting wash buffer into each well with a squirt bottle, or by pipetting 300 µL of wash buffer into each well and then discarding the liquid over a sink. After each wash, the plate should be thoroughly blotted on paper towels before turning upright. If using a plate washer, blotting is still recommended after the last wash.

Step 8: Add 200 µL of TMB Substrate Solution to each well with a multichannel pipette.

Step 9: Mix on a plate rotator for 5 minutes at 500 rpm and incubate the plate in the dark (covered) at room temperature for an additional 25 minutes.

Step 10: Add 50 µL of Stop Solution with a multichannel pipette.

Step 11:

- Mix on a plate rotator for 3 minutes at 500 rpm. If green color remains, continue mixing until green color turns to yellow. Be sure all wells have turned yellow.
  Caution: Spillage may occur if mixing speed exceeds 600 rpm.
- Wipe off bottom of plate with a water-moistened, lint-free cloth and wipe dry.
- Read in a plate reader at 450 nm. Read plate within 10 minutes of adding Stop Solution. (For best results, a secondary filter correction at 620 to 630 nm is recommended.)

Quality Control

The Salimetrics' High and Low Estradiol Controls should be run with each assay. The control ranges established at Salimetrics are to be used as a guide. Each laboratory should establish its own range. Variations between laboratories may be caused by differences in techniques and instrumentation.

Calculations

1. Compute the average optical density (OD) for all duplicate wells.
2. Subtract the average OD for the NSB wells (if used) from the OD of the zero, standards, controls, and saliva samples.
3. Calculate the percent bound (B/Bo) for each standard, control, and saliva sample by dividing the OD of each well (B) by the average OD for the zero (Bo). (The zero is not a point on the standard curve.)
4. Determine the concentrations of the controls and saliva samples by interpolation using data reduction software. We recommend using a 4-parameter non-linear regression curve fit.
5. Samples with Estradiol values greater than 32 pg/mL should be diluted with HS Estradiol Assay Diluent and rerun for accurate results. If a dilution of the sample is used, multiply the assay results by the dilution factor.

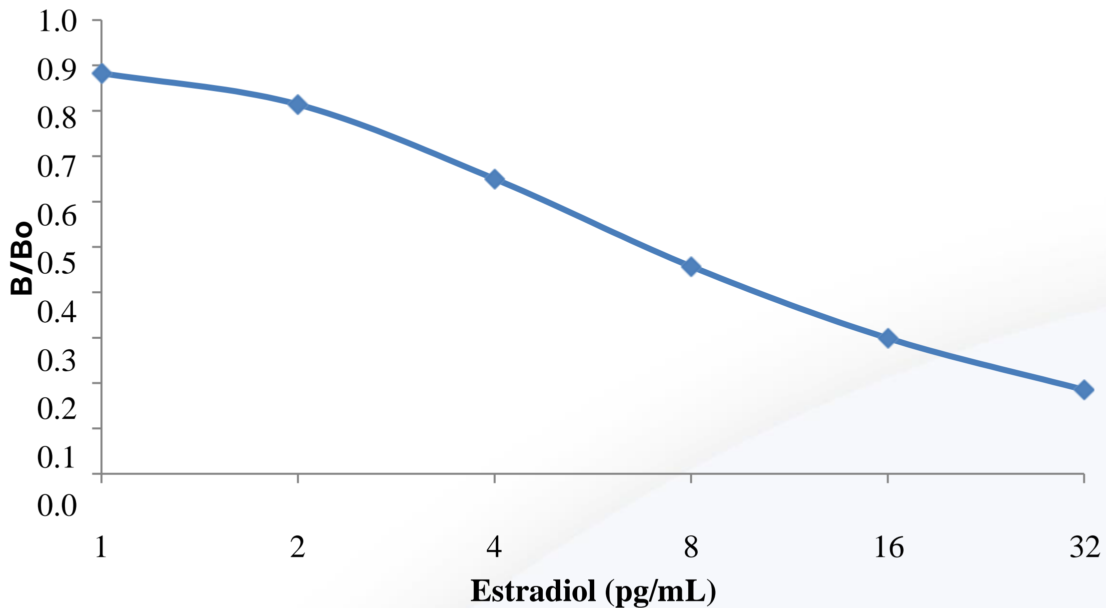
A new Standard Curve must be run with each full or partial plate.

Typical Results

The results shown below are for illustration only and should not be used to calculate results from another assay.

Well	Standard	Average OD	B	B/Bo	Estradiol (pg/mL)
A1,A2	S1	0.183	0.174	0.185	32
B1,B2	S2	0.290	0.280	0.299	16
C1,C2	S3	0.438	0.429	0.457	8
D1,D2	S4	0.619	0.609	0.650	4
E1,E2	S5	0.773	0.764	0.814	2
F1,F2	S6	0.837	0.828	0.883	1
G1,G2	Bo	0.947	0.937	NA	NA
H1,H2	NSB	0.009	NA	NA	NA

Example: HS 17β-Estradiol 4-Parameter Curve Fit



Limitations

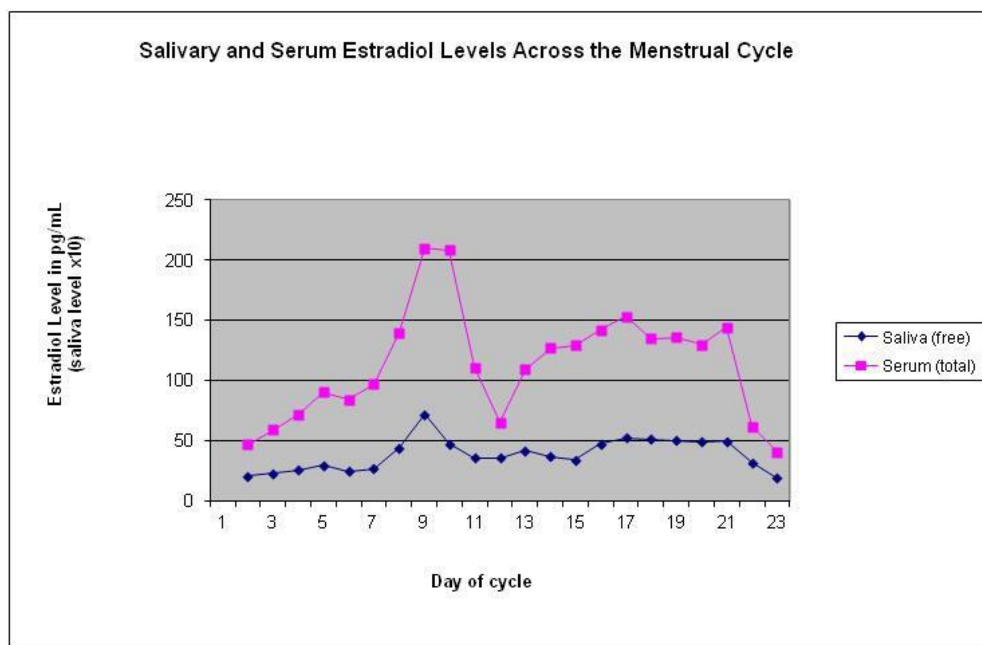
- Samples with Estradiol values greater than 32 pg/mL should be diluted with HS Estradiol Assay Diluent and rerun for accurate results. To obtain the final Estradiol concentration, multiply the concentration of the diluted sample by the dilution factor.
- A pH value should be obtained on samples that appear yellow or purple after the diluted conjugate solution is added and the plate is mixed (Step 6). Samples with pH values $\leq$ 5.0 or $\geq$ 9.0 should be recollected.
- See "Specimen Collection" recommendations to ensure proper collection of saliva specimens and to avoid interfering substances.
- Samples collected with sodiumazide are unsuitable for this assay.
- Any quantitative results indicating abnormal Estradiol levels should be followed by additional testing and evaluation.

Salivary Estradiol Example Ranges*

Pre-menopausal Adult Women	N	Mean (pg/mL)	Standard Deviation (pg/mL)
Follicular	20	1.35	0.80
Mid-Cycle	20	2.97	1.58
Luteal	20	2.56	0.84

*To be used as a guide only. Each laboratory should establish its own range.

Example of the Variation of Estradiol Levels during the Menstrual Cycle of One Woman



HS Salivary 17β-Estradiol EIA Kit Performance Characteristics

Precision

The intra-assay precision was determined from the mean of 14 replicates each.

Saliva Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	Coefficient of Variation (%)
High	14	20.2 6	1.42	7.0
Mid	14	7.24	0.45	6.3
Low	14	3.81	0.31	8.1

The inter-assay precision was determined from the mean of average duplicates for 10separate runs.

Saliva Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	Coefficient of Variation (%)
H	10	24.6 2	1.47	6.0
L	10	4.76	0.42	8.9

Recovery

Five saliva samples containing different levels of endogenous Estradiol were spiked with known quantities of Estradiol and assayed.

Saliva Sample	Endogenous (pg/mL)	Added (pg/mL)	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1	2.92	20.48	23.40	23.84	101. 9
2	4.68	13.65	18.33	17.91	97.7
3	3.80	3.20	7.00	6.78	96.9
4	5.41	20.48	25.89	28.2	108. 9
5	3.69	3.20	6.89	8.26	120. 0

Sensitivity

The lower limit of sensitivity was determined by interpolating the mean optical density minus 2 SDs of 10 sets of duplicates at the 0 pg/mL level. The minimal concentration of Estradiol that can be distinguished from 0 is 0.1 pg/mL.

Correlation with Serum

The correlation between serum and saliva Estradiol in females was determined by assaying 11 matched samples. Samples were screened for pH and blood contamination. The magnitude of the serum-saliva correlation, $\underline{r}(9)= 0.80$, $\underline{p} = <0.001$, is consistent with the literature (4,15,19).

Sample Dilution Recovery
Four samples were serially diluted with HS Estradiol Assay Diluent and assayed.

Saliva Sample	Dilution Factor	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1			28.98	
	1:2	14.49	13.57	93.7
	1:4	7.25	7.24	99.9
	1:8	3.62	3.73	103.0
2			23.84	
	1:2	11.92	12.03	100.9
	1:4	5.96	5.56	93.3
	1:8	2.98	3.60	120.8
3			6.78	
	1:2	3.39	3.07	90.6
	1:4	1.70	1.70	100.0
4			8.54	
	1:2	4.27	4.55	106.6
	1:4	2.14	1.93	90.2

Antibody Specificity

Compound	Spiked Concentration (ng/mL)	% Cross-reactivity in HS 17β-Salivary Estradiol EIA
Estriol	10	0.234
Estrone	1	1.276
Progesterone	100	ND
17 α-Hydroxyprogesterone	1000	ND
Testosterone	1000	ND
Cortisol	1000	ND
DHEA	1000	ND
Androstenedione	1000	ND
Aldosterone	1000	ND
Cortisone	1000	ND
11-Deoxycortisol	1000	ND
21-Deoxycortisol	1000	ND
Dexamethasone	1000	ND
Triamincinolone	1000	ND
Corticosterone	1000	ND
Prednisolone	1000	ND
Prednisone	100	0.016
Transferrin	1000	ND
Ethynodiol diacetate	1000	ND
Ethynylestradiol	10	0.189

ND = None detected (<0.004)

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"Seller warrants that all goods sold hereunder will be free from defects in material and workmanship. Upon prompt notice by Buyer of any claimed defect, which notice must be sent within thirty (30) days from date such defect is first discovered and within three months from the date of shipment, Seller shall, at its option, either repair or replace the product that is proved to Seller's satisfaction to be defective. All claims should be submitted in written form. This warranty does not cover any damage due to accident, misuse, negligence, or abnormal use. Liability, in all cases, will be limited to the purchased cost of the kit.

It is expressly agreed that this limited warranty shall be in lieu of all warranties of fitness and in lieu of the warranty of merchantability. Seller shall not be liable for any incidental or consequential damages that arise out of the installation, use or operation of Seller's product or out of the breach of any express or implied warranties."

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Updated: April 19, 2019

Appendix I - **Godin Leisure-Time Exercise Questionnaire**

Godin Leisure-Time Exercise Questionnaire

INSTRUCTIONS

In this excerpt from the Godin Leisure-Time Exercise Questionnaire, the individual is asked to complete a self-explanatory, brief four-item query of usual leisure-time exercise habits.

CALCULATIONS

For the first question, weekly frequencies of strenuous, moderate, and light activities are multiplied by nine, five, and three, respectively. Total weekly leisure activity is calculated in arbitrary units by summing the products of the separate components, as shown in the following formula:

Weekly leisure activity score = (9 × Strenuous) + (5 × Moderate) + (3 × Light)

The second question is used to calculate the frequency of weekly leisure-time activities pursued "long enough to work up a sweat" (see questionnaire).

EXAMPLE

Strenuous = 3 times/wk

Moderate = 6 times/wk

Light = 14 times/wk

Total leisure activity score = (9 × 3) + (5 × 6) + (3 × 14) = 27 + 30 + 42 = 99

Godin Leisure-Time Exercise Questionnaire

1. During a typical **7-Day period** (a week), how many times on the average do you do the following kinds of exercise for **more than 15 minutes** during your free time (write on each line the appropriate number).

Times Per Week

a) STRENUOUS EXERCISE (HEART BEATS RAPIDLY) ________

(e.g., running, jogging, hockey, football, soccer, squash, basketball, cross country skiing, judo, roller skating, vigorous swimming, vigorous long distance bicycling)

Appendix J - **CHESS Study Data Collection Notes**

Baseline CHESS Study (August 30th – September 14th, 2018)
In the first two weeks of the academic semester, 100 students were approached and asked to fill out the SSI to give us an overall stress distribution curve. The surveys were filled out anonymously, and one student wrote on the back of a questionnaire a concerning self-harming statement. I had no method of tracking down which student it was to make sure they had resources to assist them. This made me realize that I was entering a potential area of vulnerability for students and I wanted to be better prepared. I joined a Student Wellness volunteer program for the year providing me with suicide intervention training, knowledge regarding resources on campus as well as experience volunteering in U of C clinic. I committed to this volunteer position throughout the duration of the academic year, which help enriched my trainee experience.

Participants (August 30th, 2018 – Rolling recruitment)
We conducted recruitment through a variety of approaches with rolling intake to the study, yet still struggled with numbers. Posters were the first step and attracted a few participants. RS, the honours student on the project, also did a good job recruiting having 3 or 4 colleagues come onto the project as participants. Many potential participants went through the initial stages of recruitment, but decided not to partake as they believed the study was too long or too labour intensive. We also had a number of initial recruits who decided to leave on exchange for Winter 2019 and thus, we could not keep them as participants.

I also approached university athletic teams including: rowing, squash, field hockey and track and field. I thought this would be a very positive source of recruitment given the HRV and DXA results they would receive. However, from all 4 teams, only 5 individuals agreed to participate.

There were two social media publications regarding the CHESS study to aid in awareness and recruitment. Although I believe these were positive items, they only recruited two participants, yet I received hundreds of emails from ineligible individuals (too old, not students, etc). This was very time consuming to sort through and respond to each.

The rolling recruitment was positive as it allowed us to maximize participation. The process got smoother as each new participant completed intake. I began to learn the common questions/confusions and started to address them with new participants from the beginning. A challenge was keeping track of what stage people were at in the study as they all entered and progressed at different speeds.

I believe from talking to the potential participants that the number and diversity of data collection tasks on the study served as a deterrent for participation. Individuals did not mind either the salivary samples and the stress questionnaire or the HRV, but by the time all components were included (saliva, SSI, HRV, DXA and dietary journal), the participants felt it was too much and were overwhelmed.

Dropouts

On November 21st, 2018 the first drop-out occurred due to "being too busy to complete all the requirements of the study" – R1. On January 7th, 2019 the second drop-out occurred due to "already falling behind on all the components of the study and anticipating a worse semester" – R2 (interestingly, R2 was the only participant who was a result of the social media postings, which leads to questioning how effective these are in recruiting for longitudinal studies). On January 10th, 2019 the third drop-out occurred due to "being too busy to complete all requirements and having constant trouble using the HRV4Training application. It consistently told her to repeat her data collection." -R3. Throughout January, 2 individuals stopped responding to information and failed to continue to complete data collection, thus they have only 1 semester of data, but will still be used in analysis. No reason was given directly to me communicating the drop-out.

<u>Data Collection</u>
Missing Data: Many participants travelled out of country during the Holiday season and were therefore, unable to complete data collection at this time. The occasional participant missed their data collection on day 21 and as a result, that cycle had to be skipped. One participant did not want any type of body composition testing performed, and as a result, we are missing DXA results for her.

Participant Independence: I think the project was well set up in terms of its non-invasiveness for the participants. They rarely had to come into the lab and all testing was very passive for them; however, this also meant that participants had low compliance in getting things done. For example, on average over the first month, HRV collection statistics usually showed that 100% of participants collected data once per week, however, the average daily percentage of participants completing measures often fell between 60-70%. Some participants also tended to not report menses right away and then we had to back track to establish day 0, which potentially reduces accuracy of our timeline.

<u>Measurement Tools</u>
HRV4Training: Initial complications with this application delayed getting the process started. We ended up having 3 participants purchase the app themselves due to lack of clear communication by the owner, which resulted in problems connecting participants to the coaching profile of the lab later in the study. Initially, we had discussed taking HRV measure once per week in each participant, so it was difficult for the first few participants when I then had to return and tell them to take in every day. After this communication was cleared up all recruited participants were instructed to measure HRV every morning. The app worked very well for iPhone users. The connection and data quality were strong and we received good feedback from the participants for the first few months. Despite the company explicitly stating equal compatibility with android users, we had difficulty with data collection and transmission via the android users and received negative feedback from these participants as frustrations grew for them and us. Into January and moving forward in 2019, more and more participants struggled to use the HRV4Training App (including iPhone users) which consistently reported insufficient quality of data, forcing the participants to repeat the measure too many times and they gave up. This substantially reduced compliancy to the protocol. RS also had difficulties with exporting data for usage in her honours project, and as a result, a great deal of time was spent by me on this process.

eaTracker: This application was chosen based on the advice of a well-known researcher in Kinesiology and added to the study later in the proposal process; in hindsight I feel it may not have been the right choice. There were three main problems that arose using this application: participants consistently complained that their foods were not available within the application (R4: "Also, can I please add that this food tracking app is a nightmare. It literally has NOTHING on it, and makes tracking food so hard and inaccurate. It doesn't even have smoothie as an option! I'm gonna cry"), there was no clear way to transport information from each personal account to the research team and the summary outputs that were available for printing and export did not break down dietary intake by meal, which did not suit the project goal of analyzing breakfast information. It would have been easier to ask for written dietary journals which could have been emailed in and then manually entered into a dietary intake system. This application created more work and confusion than benefits.

Salivary Samples: As far as I can tell, salivary samples worked well for participants based on the feedback. I worry about if the participants kept them frozen after taking the sample, but based on self-report, they did. Some participants repeatedly did not fill out the appropriate forms with their samples, but I did my best to make sure I got everything completed when the samples were returned. Another note is that on January 7th, 2019 there was a power outage in the HPL. It only lasted 15 minutes, so the freezer remained cold, however, there may have been a slight temperature change. In total, 6 tubes were not usable in the Labrix analysis. Three tubes had traces of blood and three tubes had leakage during transport. As a result, these profiles were created with one less sample than the others.

DXA: I noticed with the younger students it was extremely difficult to have participants remove all sources of metal for the scans. These female students have a large number of piercings, metal rods etc., that could not be removed. The options were to remove the participants from the study or perform the scans anyways. In all cases, we removed as much metal as possible, but some items remained during the DXA scan. This may impact accuracy of the results and may be something to consider in future use of the DXA machine with young women in studies.

The Student-Life Stress Inventory (SSI): I think this questionnaire served its purpose well. I had positive feedback for participant usage. It was simple and inclusive. Half way through data collection (late January), it was suggested that the SSI might not have been the correct stress questionnaire for this study due to its lack of prior use in repeated measures studies. I reached out and spoke to Dr. Meghan McDonough who agreed it may not have been the strongest choice, but believed due to the nature of the questionnaire (state vs trait questions) it was an OK measuring tool to complete the CHESS study. However, it is important to note that a different questionnaire should be used moving forward.

Dates with specific experiences
February 6th, 2019: First shipment was sent out to Labrix.
February 11th, 2019: I spoke to Meghan McDonough regarding the SSI validity and applicability for repeated measures.
February 15th, 2019: Abstract was submitted to ECSS.

February 22nd, 2019: I presented about CHESS to West Island College in Calgary health seminar series.
March 8th, 2019: DXA computer hardware broke and I was forced to efficiently solve this problem before my first participant arrived at 10am.
March 12th, 2019: Other HPL user overbooked my DXA slot to measure rats – it was unprofessional, and I was forced to ask him to leave, recalibrate the machine and clean it while my participant waited. She was concerned to see rats on the machine before herself.
March 18th, 2019: I presented the material to the MKIN lecture series.
March 20th, 2019: I completed my first 3MT presentation for CHESS.
April 3rd, 2019: I finished 2nd in the 3MT ucalgary finals and was approached by the Chancellor and Provost of the University to discuss my research.
May 2nd, 2019: CHESS research was presented via Operation Minerva at the HPL.
May 9th & 10th, 2019: CHESS research knowledge translation was presented at the UofT BOK Kinesiology Conference.
May 15th, 2019: Statistical analysis meeting occurred with statistician Dr. Tak Fung.

Team Collaboration

Throughout this process, I had three undergraduate students working with me: RS (honours student), DG (Markin USRP student) and SI (volunteer). RS focused mainly on the HRV data in correlation with SSI and Salivary Sample results, while DG focussed mainly dietary intake in correlation with the SSI. Having all aspects made for a much more inclusive and interesting study. It was a great learning experience for me acting as the research coordinator and overseeing the undergraduate students day-to-day. It was also one of the most time-consuming aspects of the study for me.

I assisted RS in many aspects of her honours course work including proposal, presentation, data analysis and statistics plans and spent approximately an hour per day reviewing her HRV data collection. DG struggled with tracking the dietary intake. The USRP did not start until November which complicated the situation as the study was already two months into data collection. Over the duration of the study, I think her grasp of data collection improved. My leadership and management skills also vastly improved with this experience.

On January 15th, 2019, SI joined the CHESS team. She was looking to gain research experience. She assisted with data entry of SSI and DXA results into an excel spreadsheet designed by myself. She also assisted in preparing the Labrix samples for shipment which is a lengthy process making sure all sample documents and forms are complete and as accurate as possible.

Throughout my master's duration, I collaborated on the ROWERS study with TVD and vice versa. She also had an honours student, KS and as a team we worked on presentation skills, scholarship application writing and putting forth the strongest representation of our small lab. These skills, all valuable, contributed to an enriching and very busy Masters experience. I learned a great deal from Dr. Tish Doyle-Baker who is a demanding supervisor in a very positive sense. She encouraged building upon the foundation of my critical thinking skills while fostering improvement in writing and study design. For a Master's student I feel she gave me immense amounts of freedom and responsibility, which was intimidating initially, but allowed for an

extremely enriching process. I am very grateful to have been overseen by her throughout the CHESS project.

Appendix K - **GLM Cortisol Results**

NH and EH Cortisol Measures over Time (N=19)

Group	Month	Time of day	Mean, SE (nmol)	95% Wald CI
NH	Nov	CAR	17.34 ± 5.4	6.78 – 27.90
	(n=3)	Noon	1.68 ± 0.8	0.07 – 3.28
		Evening	2.66 ± 0.2	2.23 – 3.10
		PM	2.09 ± 0.5	1.07 – 3.12
	Dec	CAR	7.75 ± 2.0	3.88 – 11.61
	(n=4)	Noon	4.99 ± 1.6	1.82 – 8.16
		Evening	1.93 ± 0.1	1.80 – 2.06
		PM	2.88 ± 0.4	2.00 – 3.77
	Jan	CAR	18.79 ± 8.1	2.92 – 34.66
	(n=5)	Noon	2.86 ± 0.6	1.65 – 4.07
		Evening	2.59 ± 0.3	2.06 – 3.11
		PM	2.10 ± 0.7	0.75 – 3.46
	Feb	CAR	9.56 ± 1.3	7.02 – 12.11
	(n=5)	Noon	4.30 ± 0.6	3.09 – 5.52
		Evening	1.94 ± 0.5	0.98 – 2.90
		PM	2.47 ± 0.6	1.21 – 3.72
	Mar	CAR	12.10 ± 1.7	8.71 – 15.49
	(n=6)	Noon	4.23 ± 0.4	3.39 – 5.07
		Evening	2.74 ± 0.4	1.90 – 3.59

		PM	6.01 ± 4.3	-2.35 – 14.37
EH	Nov	CAR	13.18 ± 2.2	8.92 – 17.44
	(n=5)	Noon	2.67 ± 1.0	0.64 – 4.70
		Evening	2.09 ± 0.03	1.43 – 2.75
		PM	2.62 ± 0.9	0.82 – 4.42
	Dec	CAR	12.94 ± 2.0	9.03 – 16.85
	(n=5)	Noon	5.29 ± 0.9	3.58 – 6.99
		Evening	2.00 ± 0.3	1.35 – 2.66
		PM	3.99 ± 2.5	-0.84 – 8.81
	Jan	CAR	10.06 ± 1.2	7.63 – 12.48
	(n=7)	Noon	4.52 ± 1.1	2.30 – 6.74
		Evening	3.76 ± 0.7	2.39 – 5.12
		PM	1.96 ± 0.2	1.51 – 2.41
	Feb	CAR	9.99 ± 0.9	8.16 – 11.82
	(n=8)	Noon	6.09 ± 2.1	1.93 – 10.24
		Evening	3.74 ± 1.9	0.09 – 7.38
		PM	3.79 ± 2.3	-0.76 – 8.35
	Mar	CAR	11.01 ± 2.0	7.08 – 14.94
	(n=7)	Noon	2.97 ± 0.9	1.20 – 4.74
		Evening	3.50 ± 0.5	2.46 – 4.55
		PM	2.20 ± 0.3	1.66 – 2.74

Appendix L - **CHESS Study Budget**

Item	Cost per Unit ($)	Number of Units	Total Costs ($)
Pg, E, C x4	65.00	55	3757.00
Pg, E, C x3	55.00	3	165.00
HRV Application	13.99	20	279.80
Total Budget			**4201.80**

Appendix M - **Raw Data**

Table 6.1. Characteristic Data

ID	Group	Baseline SSI	LTAQ
NH1	1	138	43
NH2	1	179	46
NH3	1	134	110
NH4	1	143	91
NH5	1	158	64
NH7	1	151	45
NH8	1	134	60
NH9	1	123	49
NH10	1	145	80
EH1	2	113	34
EH2	2	130	61
EH3	2	110	58
EH4	2	130	
EH5	2	152	96
EH6	2	160	91
EH7	2	117	65
EH8	2	154	67
EH9	2	174	65
EH10	2	170	64

Table 6.2 DXA Data

ID	Group	DXA1 T-Score	DXA1 BF%	DXA1 Z-Score	Mnths btwn tests	DXA2 T-Score	DXA2 BF%	DXA2 Z-Score
NH1	1							
NH2	1	0.8	32	0.8				
NH3	1	1.8	19	1.8	5	1.6	19.9	1.7
NH4	1	0.8	25	0.8				
NH5	1	-0.5	34.3	-0.5	4	-0.3	32.1	-0.3
NH7	1	0.7	25.7	0.8	2	0.8	26.8	0.8
NH8	1	0.5	18.9	0.5	4	0.8	17.3	0.8
NH9	1	0.9	25.3	0.9	1	1	25.3	1
NH10	1	0.1	35	0.2				
EH1	2	0.7	30.2	0.8	5	0.8	26.7	0.9
EH2	2	0.6	26.8	0.6	3	0.5	25.5	0.6
EH3	2	0.6	25.6	0.6	3	0.8	26.5	0.8
EH4	2	-0.3	17.6	-0.2				
EH5	2	1	32.4	1	4	0.7	33.3	0.7
EH6	2	0.7	33.5	0.7	4	0.7	34	0.7
EH7	2	-0.9	34.4	-0.8	4	-1	33.4	-0.9
EH8	2	0.3	30.5	0.4	4	0.5	30.8	0.6
EH9	2	2.2	25.2	2.2	3	2.2	25	2.3
EH10	2	0.5	31.2	0.6				

Table 6.3. Fall Semester Data

		October								November								December							
ID	Group	SSI 1	CAR 1	C NOON 1	C EVENING 1	C PM 1	E 1	P 1	E:P 1	SSI 2	CAR 2	C NOON 2	C EVENING 2	C PM 2	E 2	P 2	E:P 2	SSI 3	CAR 3	C NOON 3	C EVENING 3	C PM 3	E 3	P 3	E:P 3
NH1	1																	144	7	6.7	1.7	2.6	3.2	262	81.9
NH2	1	150	29	4	3.4	11	1.1	68	61.8	175	6.3	1.6	2.5	2.1	1.3	117	90								
NH3	1	119	8.3	1.9	2	4.4	0.9	175	194									89	13	9	2	2.4	1.3	148	114
NH4	1									153	24	1.8	3.2	3.2	1.7	43	25.3								
NH5	1									137	13	3.5	2.3	1	1.1	182	166								
NH7	1																		8.8	2.8	2	2.1	1.1	176	160
NH8	1																	103	13	1.2	2	4.4	1.4	271	194
NH9	1																								
NH10	1																								
EH1	2	113	7.4	5	4	1.3	0.9	159	177	138	7.6	5.5	1.6	1.3	1.1	144	131								
EH2	2																	123	17	2.5	2.9	1.1	1.3	34	26.2
EH3	2																	122	9.5	5.5	1.9	1.4	1.3	48	36.9
EH4	2																	141	20	2	0.66	15	0.9	58	64.4
EH5	2									120	12	5.8	2.3	3.6	2.5	65	66								
EH6	2									164	23	5.2	1.5	0.66	0.6	88	147								
EH7	2									107	14	3.6	3.5	6.2	1.2	91	75.8								
EH8	2									125	8.4	4.4	1.6	1.4	1.2	19	15.8	112	9	6.9	2.1	1.3	0.9	67	74.4
EH9	2																	163	8.5	5.1	2.2	1.2	1.5	112	74.7
EH10	2																								

Table 6.4. Winter Semester Data

		January								February								March							
ID	Group	SSI 4	CAR 4	C NOON 4	C EVENING 4	C PM 4	E 4	P 4	E:P 4	SSI 5	CAR 5	C NOON 5	C EVENING 5	C PM 5	E 5	P 5	E:P 5	SSI 6	CAR 6	C NOON 6	C EVENING 6	C PM 6	E 6	P 6	E:P 6
NH1	1																								
NH2	1																								
NH3	1	77	56	2.5	2.5	4.9	1.2	192	160	80	18.4	2.67	3.39	5.04	1.4	245	175	100	11.6	4.57	2.59	2.48	0.8	224	280
NH4	1	178	6	5.8	2.7	2.5	1.3	16	12.3																
NH5	1	153	14	2.8	2.7	0.91	1.4	202	144	153	11.9	4.9	0.85	0.66	1.3	199	153	152	13.3	3.88	3.06	29.3	1.5	211	141
NH7	1	95	7.7	2	1.6	0.74	0.9	175	194	138	8.35	2.42	1.21	1.79	0.8	185	231	143	10.2	2.26	0.77	0.38	0.7	128	183
NH8	1									122	11	4		2.4	1.8	168	93.3	145	12.1	3.22	3.53	2.26	1.2	315	263
NH9	1	114	12	2.8	3.5	1.6	1.7	82	48.2									138	6.39	4.93	4.16	0.99	1.2	144	120
NH10	1									141	9.98	3.17	2.15	2.31	1.1	184	167	153	21	3.58	2.39	0.68	1.3	167	129
EH1	2	156	6.9	2.5	2.5	0.99	0.6	169	282	112	9.07	1.74	0.33	0.36	1.3	102	78.5	103	6.72	1.54	4.08	2.34	1	192	192
EH2	2									76	10.4	3.08	2.59	1.93	0.9	127	141	92							
EH3	2	160	7.3	5.5	1.7	2.3	1.6	51	31.9									154	16.5	3.22	3.5	1.15	1.7	141	82.9
EH4	2																								
EH5	2	130	12	9.2	6.8	2.5	2	141	70.3	130	12.7	14.9	4.68	2.86	1.7	212	125	146	14.1	10.1	4.77	2.97	2.1	236	112
EH6	2	200	15		3.2	1.4	0.9	69	76.7	197	8.38	17.6	15.3	18.7	1	108	108	160	3.66	3.39	2.97	3.08	0.7	47	67.1
EH7	2	92	8	5.6	3.6	2.9	1.4	75	53.6	95	15.5	2.28	1.35	1.51	2.4	140	58.3	114	16.4	2.92		2.64	1.9	157	82.6
EH8	2	120	14	5.2		1.8	1.8	43	23.9	132	9.26	2.15	0.99		0.9	95	33.3	152	15.1	2.31	1.07	1.37	0.9	149	52.2
EH9	2	162	7.1	0.7	5.2	1.9	1.2	47	39.2	159	7.28	3.97		0.85	1.4	71	50.7	176	4.46	2.06	4.93	1.76	1.6	93	58.1
EH10	2									158	6.95	1.4	0.93	0.34	1.2	137	114								