GOLDENSTEIN, SAMANTHA J. Ph.D. Energy Availability and Sex Steroid Hormones in Physically Active Females. (2023)
Directed by Dr. Laurie Wideman. 215 pp.

Sex steroid hormones (i.e., estrogen, progesterone) are major determinants of a women's health status and play a role in almost every physiological system, including reproductive, endocrine, urinary, nervous, immune, musculoskeletal and cardiovascular. Physically active females have lower estrogen and progesterone when compared to inactive individuals, yet exercise alone does not appear to decrease hormones but alters hormones as a component of energy availability. Energy availability is the amount of energy left after subtracting the energy cost of exercise relative to fat free mass (FFM) from energy intake. When energy availability is inadequate (i.e., low energy availability (LEA)), disruptions to hormonal and metabolic systems occur that can lead to performance decrements and serious psychological and physiological (i.e., menstrual dysfunction) health outcomes. Furthermore, stress can disrupt estrogen and progesterone but how stress affects the relationship between energy availability and sex steroid hormones is unknown. Most research surrounding this topic includes only highly competitive, elite level female athletes and little is known about how energy availability alters hormone levels in physically active females. Since low sex steroid hormone concentrations and LEA are associated with serious health risks, further investigation into the association of energy availability and menstrual cycle hormones in physically active females is warranted. Thus, the purpose of this dissertation was to examine the relationship between energy availability and sex steroid hormones in active females across the menstrual cycle. Healthy, exercising females (n=21; age 21.3 ± 3.1 years) not on oral contraceptives completed measures over two menstrual cycles. Daily saliva measurements were taken across both menstrual cycles to create hormonal profiles of estrogen and progesterone. Energy availability was measured twice within one menstrual cycle, with energy intake recorded for seven days at two timepoints. Participants were all physically active and were asked to continue exercising normally and to record all

exercise with a heart rate monitor. The first timepoint (T1) started during menses between day (D) 2-4 and the second timepoint (T2) started between 5-8 days post ovulation. A laboratory visit occurred on the first day of each timepoint, where resting metabolic rate and body composition were measured. Stress was measured with the Acute Recovery and Stress Scale at the beginning and end of each timepoint. Area under the curve (AUC) and range (i.e., difference in minimum and maximum values) for estrogen and progesterone for T1 and T2 was used for analyses. Most of the active females (71%, n = 15) were in a reduced energy state and 23% (n = 6) had subclinical menstrual dysfunction. Energy intake and energy availability remained constant across the two timepoints despite that estrogen and progesterone were significantly different ($p = .003$, $p = .001$, respectively). When the components of energy availability and hormones were assessed, progesterone range was positively associated with FFM (T1 $p = .015$, $r=.537$; T2 $p = .001$, $r = .674$) and RMR (T2 $p = .005$, $r = .605$) yet T2 progesterone range, FFM, and RMR were all negatively associated with energy availability ($p = .032$, $r = -.479$; $p = .001$, $r = -.672$; $p = .009$, $r = -.558$). Energy intake was correlated with the progesterone to estrogen ratio (P:E2) ($p = .026$, $r= .321$, 95% CI [0.04, 0.55]), but not progesterone or estrogen alone. The results also demonstrated that estrogen, progesterone, and the estrogen progesterone product in T1 exhibited a negative relationship with T2 energy availability (($\beta = -.36$, $p = .009$; $\beta = -.37$, $p = .008$; $\beta = -.31$, $p = .029$), in active females across a single menstrual cycle. In addition, stress and recovery do not moderate the relationship between hormones and energy availability within a timepoint or across timepoints of one menstrual cycle even though a stress subscale, negative emotional state, was significantly higher post ovulation towards the end of the cycle while recovery and other stress scales remained constant ($F (3, 54) = 7.07$, $p = .000$). These data suggest that physically active females are at risk for inadequate energy availability and subclinical menstrual dysfunction. Estrogen and progesterone affect energy intake at the beginning of the cycle and energy

availability across timepoints but do not appear to be altered by stress and recovery. A higher progesterone to estrogen ratio was associated with higher energy intake during T1. Furthermore, higher estrogen and progesterone at the beginning of the menstrual cycle are associated with lower energy availability post ovulation. These data highlight the importance of including physically active females in future research on energy availability and emphasizes issues with energy availability are present in physically active females, not just elite athletes. Further investigations are needed to fully elucidate the relationship between energy availability, estrogen and progesterone.

ENERGY AVAILABILITY AND SEX STEROID HORMONES IN PHYSICALLY ACTIVE FEMALES

by

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A Dissertation
Submitted to
the Faculty of The Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Greensboro

2023

Approved by

Dr. Laurie Wideman
Committee Chair

DEDICATION

To Dad.

Thank you for the sacrifices you made as a single father and for your love and support. This journey would not have been possible without you. My Dad taught me to push my limits and not to fear failure because I knew if I failed, he would always be there to support me. He was my rock and gave me the confidence to go outside my comfort zone. Although he did not get to see this part of my life, he has been with me every step of the way and I would not have been able to accomplish this without him.

APPROVAL PAGE

This dissertation written by Samantha J. Goldenstein has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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June 12, 2023

Date of Acceptance by Committee

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ACKNOWLEDGMENTS

I would like to thank Dr. Wideman for her support and encouragement over the last four years. She listened to all of my ideas and never told me no. No matter what the idea was, she supported me and assisted me in making my ideas a reality. Her mentorship allowed me to follow my passion and help shape my future.

I would like to thank my committee members Dr. Dollar, Dr. McNeil, Dr. Shriver, and Dr. Adams. Without their help, support, and guidance through this process, none of this work would have been possible.

To Dave, my friends, and family. Thank you for the support and encouragement, it takes a village and I could not have done this alone. Thanks for putting up with my always changing schedule, early mornings, and short weekends. I am excited to share my next chapter with you.

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CHAPTER I: INTRODUCTION

Sex steroid hormones (i.e., estrogen, progesterone) are major determinants of premenopausal women's health status. Estrogen (E2) is the predominate female sex hormone and plays a role in almost every physiological system, including reproductive, endocrine, urinary, nervous, immune, musculoskeletal and cardiovascular. E2 serves a protective role from multiple diseases in premenopausal women, therefore disruption of E2 is associated with diseases such as cancer, cardiovascular disease, osteoporosis, neurodegenerative diseases, and metabolic disorders (Prossnitz & Barton, 2011). Progesterone regulates not only reproductive function but the nervous system and vessels as well. Further research is needed to fully understand the role of endogenous progesterone but research has demonstrated a neuroprotective effect in traumatic brain injury, stroke, and myelin repair (Sitruk-Ware & El-Etr, 2013). Additionally, the estrogen to progesterone ratio can influence core body temperature, sleep, and fluid volume (Giersch et al., 2021; Grant et al., 2020).

Proper menstrual cycle function is commonly thought of as a vital sign for women's health and inadequate E2 and progesterone concentrations can disrupt the menstrual cycle (American Academy of Pediatrics et al., 2006). A 'normal' menstrual cycle ranges from 21-35 days and hormones (luteinizing hormone (LH), follicle stimulating hormone (FSH), E2, and progesterone) vary throughout the cycle, but work together in negative feedback loops to maintain proper function. Disturbances in the feedback loop can result in menstrual dysfunction, which occurs on a spectrum ranging from subclinical (i.e., luteal phase defect (LPD), anovulation) to clinical (i.e., oligomenorrhea, amenorrhea). Anovulation—failure of the ovary to release an egg in response to LH stimulation, occurs in over a third of normal menstrual cycles (Prior et al., 2015), while luteal phase defect (LPD) (i.e., luteal phase less than 10 days) affects up to 20% of women (De Souza, 2003). LPD allows ovulation to occur but due to decreased

progesterone, implantation of the egg is not supported (De Souza, 2003). Menstrual dysfunction prevalence rates are even higher in exercising women, with De Souza et al. (2010) demonstrating that 50% of exercising females experience subclinical menstrual disturbances while one third may be amenorrheic (De Souza et al., 2010a).

It is widely accepted that exercising females (i.e., engaging in physical activity for health or fitness) have lower menstrual cycle hormones (i.e., E2, progesterone) when compared to sedentary individuals (Cumming et al., 1985; De Souza et al., 1998a; Ellison et al., 1987; Fisher et al., 1986; Jasienska et al., 2006; Lehmann et al., 1993; Matthews et al., 2012; Stoddard et al., 2006). However, exercise alone does not appear to decrease menstrual cycle hormones but alters hormones as a component of energy availability with energy intake as a driving factor (Williams et al., 2001). Energy availability is the amount of energy left after subtracting the energy cost of exercise relative to fat free mass (FFM) from energy intake. When energy availability is inadequate (i.e., low energy availability (LEA)), disruptions to hormonal and metabolic systems occur that can lead to performance decrements and serious psychological and physiological health conditions (e.g., menstrual dysfunction, cardiovascular disease, decreased bone mineral density) (Mountjoy et al., 2014). While there is no accepted threshold of energy availability that induces negative health conditions, when energy availability falls below 30 kcal/kg/FFM/day, females have a 50% increased risk of menstrual dysfunction (Lieberman et al., 2018). Nonetheless, it is unclear why menstrual function is disrupted by LEA in some females while menstrual function remains normal in others.

However, other factors such as the stress of exercise should also be considered when evaluating LEA. Excess exercise stress and inadequate recovery can place an individual into nonfunctional overreaching and over training states, which have demonstrated similar symptoms to LEA (e.g., decreased estrogen and progesterone) yet energy availability and

menstrual function are rarely assessed. Since low sex steroid hormone concentrations are associated with serious health risks, further investigation into the association of LEA and menstrual cycle hormones is warranted, especially since most research on this topic includes only highly competitive, elite level women athletes and little is known about how LEA alters hormone levels in physically active females (i.e., not a professional or national level athlete). Therefore, the purpose of this study is to examine energy availability and menstrual cycle hormones in physically active females across the menstrual cycle and the following overarching questions will be addressed:

1. What is the relationship of energy availability, estrogen, and progesterone across a menstrual cycle?
2. What is the association of estrogen, progesterone, and energy availability in the follicular phase and post ovulation?
3. Do stress and recovery influence the relationship of energy availability with estrogen and progesterone?

To investigate these questions, this project has the following specific aims and research hypotheses:

Specific Aim 1

To determine the relationship of energy availability, estrogen and progesterone changes across the menstrual cycle in physically active women.

H1.1: Regardless of fitness level (VO_{2peak}), women with lower fat free mass will have lower energy availability, lower resting metabolic rate ratio, lower levels of estrogen and progesterone, and less range in hormone levels (less differences in min to max values) across one menstrual cycle and more anovulatory cycles across two menstrual cycles.

H1.2: Energy intake will have a negative correlation with estrogen concentrations, with energy intake being high when estrogen concentrations are low.

Specific Aim 2

To examine the association between energy availability, estrogen, and progesterone in the follicular and luteal phase while controlling for the previous phase in one menstrual cycle.

H2.1: Energy availability in the follicular phase will predict estrogen and progesterone concentrations post ovulation.

H2.2: Estrogen in the follicular phase will predict energy availability in post ovulation while progesterone will not affect energy availability.

Exploratory H2.3: Lower energy availability will be associated with lower estrogen and progesterone concentrations, which will be associated with greater risk of menstrual dysfunction.

Specific Aim 3

To determine if stress and recovery moderate the relationship between energy availability and estrogen and progesterone.

H3: Energy availability will predict menstrual cycle hormone concentrations, but this relationship will be stronger with increased recovery and decreased stress scores.

Delimitations and Limitations

The study design has delimitations. It is restricted to premenopausal women up to 35 years of age. This delimitation is introduced to obtain the clearest picture of the menstrual cycle hormones. Around the age of 40, women tend to enter perimenopause and estrogen and progesterone start to decrease for reasons related to age and thus, including this age group would make it difficult to determine if lower estrogen and progesterone levels were due to perimenopause or energy availability. Additionally, this study is deliminated to women who are

not on any hormonal contraceptive. There are a multitude of hormonal contraceptive options and each affects the endogenous estrogen and progesterone levels differently. Also, the study only investigates two menstrual cycles with the hope of increasing patient compliance. Lastly, menstrual cycle length was deliminated to menstrual cycles that are consistently between 21-50 days in length due to altered estrogen and progesterone levels in longer menstrual cycle lengths (i.e., oligomenorrhea, amenorrhea).

As a free-living study, there are limitations. Participant compliance could be an issue due to the length and requirements of the study. To counter this, the researchers ensured clear and concise instructions along with the importance of collecting the data as asked. The researchers also maintained weekly contact, more if necessary. The Training Peaks app served as a central data base, with energy intake and exercise data automatically uploading to the app. The allowed continuous monitoring with participant interaction to ensure the participant was being compliant. Training Peaks was also used a study calendar, with data collection reminders entered into the app. Accelerometer data was be recorded every day and provided heart rate monitors were worn with all purposeful exercise but there may be time periods the participant does not wear or is unable to wear (e.g., swimming) the watch or heart rate monitor. To counter this, participants were requested to annotate the time frame the watch or heart rate monitor was not worn and the activity that was performed during that time in Training Peaks. Saliva was collected every day, but a day may be skipped. If the sample was not collected shortly after waking, the participant was asked to skip that day and to begin again on the next saliva collection day. At home ovulation tests were performed for a certain number of days based off the length of each participant's menstrual cycle, therefore allowing the researchers to know when the ovulation testing should begin and end. The participant was expected to text the researchers a picture of the ovulation test result. If a picture is not received, the researchers

remind the participant to take the ovulation test. An energy intake entry may be missed. MyFitnessPal sends automatic reminders to the participants phone if a meal has not been logged. MyFitnessPal also automatically uploads each entry to Training Peaks, allowing the researchers to spot check and ensure compliance with the food log. Lastly, changes in the participant's normal routine could occur. The participants were asked to annotate anything abnormal (e.g., illness, life and social stressors) in Training Peaks and to inform the researchers.

CHAPTER II: REVIEW OF LITERATURE

Introduction

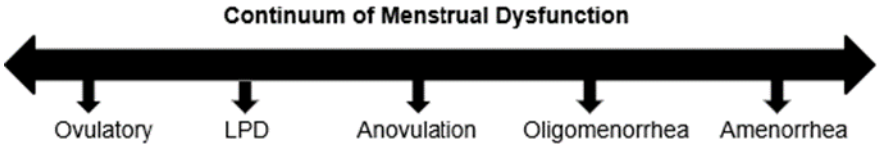
Physical activity and exercise can have many positive health benefits, but an imbalance of energy intake and energy expenditure may negate many of these positive effects. Energy availability describes the amount of energy available for metabolic functions after exercise energy expenditure (EEE) is subtracted from energy intake, and is expressed relative to fat-free mass (FFM) (Energy availability = Energy intake (kcal) – EEE (kcal)/ FFM (kg)) (Loucks et al., 1998; Mountjoy et al., 2014). When a negative balance emerges between energy intake and EEE over time, otherwise known as low energy availability (LEA), an energy conservation state is created where metabolic fuels are focused on life sustaining metabolic processes and are shifted from other systems such as the reproductive axis (De Souza, 2003).

The menstrual cycle is a complex and highly variable physiologic event controlled by the precise integration of multiple biological systems (e.g., endocrine, reproductive) (Alvergne & Tabor, 2018; Berbic & Fraser, 2013). The menstrual cycle is considered a ‘vital sign’ for women, indicative of overall health (American Academy of Pediatrics et al., 2006; Saei Ghare Naz et al., 2020) and represents an important, yet often ignored, factor in research involving females. The menstrual cycle is one component of the Female Athlete Triad, which also consists of LEA and bone health. LEA underpins the concept of the Female Athlete Triad, which illustrates the negative health consequences of LEA on menstrual function and bone mineral density (De Souza et al., 2017). LEA disrupts gonadotropin-releasing hormone (GnRH) pulsatility in the hypothalamus and the subsequent release of hormones from the pituitary and the ovaries, leading to menstrual dysfunction (Mountjoy et al., 2014). Dysfunction of the menstrual cycle occurs on a spectrum, ranging from the least severe perturbation (i.e., luteal phase defect (LPD)) to the most severe dysfunction, amenorrhea (Figure 1) (De Souza, 2003). The least

severe menstrual cycle dysfunctions (i.e., LPD, anovulation), are considered subclinical and occur within the framework of an outwardly ‘normal’ menstrual cycle, but they can potentially progress into oligomenorrhea or amenorrhea.

Menstrual cycle dysfunction is present in 6-79% of females involved in sport and varies within the specific sport and level of competition (Warren & Perlroth, 2001), yet little research is focused on exercising premenopausal females. Physically active females (i.e., not a professional or national level athlete) account for a large portion of the active population and engage in a wide variety of intensity, duration, and volume of exercise. Female exercisers may exercise less than elite athletes but may be at the same or higher risk of menstrual dysfunction since access to education and knowledgeable personnel is very limited compared to elite athletes. Knowledge of the female athlete triad and menstrual dysfunction has proven to be low amongst recreational exercisers (Folscher et al., 2015). Thus, identification of LEA and/or menstrual dysfunction may not occur, which may lead to severe long-term consequences (e.g., infertility, decreased bone mineral density).

Figure 2.1. Continuum of Menstrual Cycle Dysfunctions



Note. Menstrual dysfunctions are listed from least to most severe, starting with a normal, ovulatory menstrual cycle and ending with amenorrhea.

Menstrual Cycle Hormones

Normal menstrual cycles (i.e., 21-35 days) follow the same basic pattern in healthy females (i.e., menses, follicular, and luteal phase) but there are significant intra and inter-individual variations and cycles rarely follow the ‘typical’ 28-day cycle (Soumpasis et al., 2020).

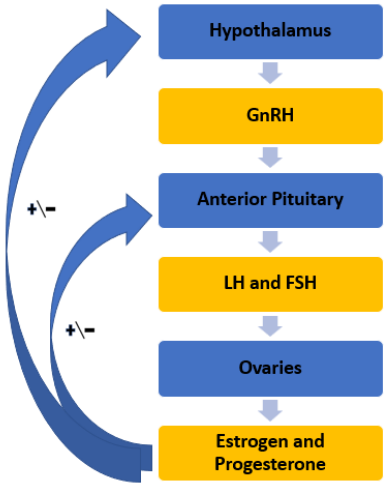
The menstrual cycle is defined as the onset of menses through to the day before the next onset of menses and is divided into the follicular and the luteal phase (Silberstein & Merriam, 2000). Ovulation—a specific event, separates the two phases with the follicular phase starting at the onset of menses and continuing to ovulation, while the luteal phase begins the day after ovulation and ends the day before the onset of menses (Yen, 1979).

The menstrual cycle is influenced by five major hormones—estrogen (E2), progesterone, luteinizing hormone (LH), follicle stimulating hormone (FSH) and GnRH—produced by the ovaries, pituitary, or hypothalamus, with feedforward and multiple nonlinear feedback loops (see Figure 2.2) (Derry & Derry, 2010). The arcuate nucleus of the hypothalamus secretes GnRH in a pulsatile fashion and is crucial to initiate ovulation and regulation of the reproductive axis (Allaway et al., 2016; Silberstein & Merriam, 2000; Suh & Betz, 1993). GnRH pulse frequency varies, slowing from one pulse every 90-100 minutes in the follicular phase when E2 is high to one pulse every 8-12 hours in the late luteal phase when progesterone is elevated (Chabbert-Buffet & Bouchard, 2002; Silberstein & Merriam, 2000). GnRH stimulates the anterior pituitary to produce and secrete gonadotropins (i.e., LH, FSH), which are reliant on the timing and concentration of GnRH (Franz, 1988). Continuing the feedforward loop, the gonadotropins stimulate production of the sex steroid hormones (i.e., E2, progesterone) in the ovaries. LH activates progesterone production in the outer ovarian theca cells while the inner granulosa cells respond to FSH to produce E2. E2 and progesterone regulate GnRH and gonadotropins in both negative and positive feedback loops, depending on the phase of the menstrual cycle (Silberstein & Merriam, 2000).

In the early follicular phase (i.e., menses), E2 and progesterone levels are low while FSH has high pulsatility that stimulates follicular growth. As the dominate follicle matures and secretes E2, FSH levels fall while E2 gradually rises throughout the follicular phase. A sharp

elevation of E2 in the late follicular phase triggers the GnRH and LH surge needed for ovulation with a small increase in FSH (Alvergne & Tabor, 2018; Chabbert-Buffet & Bouchard, 2002). The LH surge lasts for 24-48 hours, releasing the oocyte during this time. Once the oocyte is expelled, the follicular cells luteinize to form the corpus luteum and signifies the start of the luteal phase. The corpus luteum is responsible for the rise of progesterone secretion and a second, but smaller, rise of E2 while FSH and LH remain low. Progesterone levels will peak within 6-8 days after ovulation (i.e., midluteal) (Allaway et al., 2016; Yen, 1979). If no implantation occurs, the corpus luteum degenerates and decreases progesterone and E2 concentrations, triggering the shedding of the endometrium. Inhibition of the pituitary is removed and FSH will rise to begin a new menstrual cycle by stimulating follicular growth again (Allaway et al., 2016).

Figure 2.2. Schematic of Menstrual Cycle Hormones with Positive and Negative Feedback Loops



Menstrual Function

It is well-established that exercising females have decreased menstrual cycle hormones and increased menstrual dysfunction compared to sedentary females (Cumming et al., 1985; De Souza, 2003; Ellison et al., 1987; Fisher et al., 1986; Jasienska et al., 2006; Lehmann et al.,

1993; Matthews et al., 2012; Stoddard et al., 2006). Decreased GnRH frequency alters the gonadotropins and sex steroid hormone release, resulting in a spectrum of menstrual dysfunctions (see Figure 2.1) (Marshall et al., 1993). Subclinical menstrual dysfunctions (i.e., LPD, anovulatory) are difficult to diagnosis because the disturbances occur with seemingly 'normal' menstrual cycles, may not occur every menstrual cycle, and can only be confirmed with hormone measurements (i.e., LH, progesterone) (De Souza et al., 1998b). Oligomenorrhea and amenorrhea are easier to identify by a delayed or absent menses and hormone measurements are not essential for diagnosis.

Due to the complex nature of the menstrual cycle and the intra and inter-individual variations, multiple menstrual cycles should be examined in exercising females to address the issue of normal function and may include tracking two or more menstrual cycles prior to the start of, or within the framework of, the research. While more time consuming and arduous for both participant and researcher, this tracking provides confirmation of outwardly normal menstrual cycle function. In addition, confirmation of ovulation (i.e., LH, progesterone) and hormones are essential to correctly verify the menstrual cycle phase, with the outcome measures repeated in a second menstrual cycle (Elliott-Sale et al., 2021; Smith et al., 2022). Failure to confirm the menstrual cycle may result in inaccurate data as hormones influence multiple systems within the body, not just the reproductive system. Collecting data on a given day based on counting methods and assumptions of cycle length (e.g., day 14 as ovulation) is also imprecise since not all females experience 28-day cycles and even those that do, vary substantially on when ovulation occurs (Wideman et al., 2013). Participants that present with oligomenorrhea or amenorrhea should also rule out organic reasons (i.e., hyperandrogenism) that may cause menstrual dysfunction (Koltun et al., 2020).

Luteal Phase Defect (LPD)

The average luteal phase has a normal variation of 11-17 days and a progesterone peak 6-8 days after ovulation (American Society for Reproductive Medicine and the Society for Reproductive Endocrinology and Infertility, 2021; Schliep et al., 2014). LPD is defined as a luteal phase less than 10 days, inadequate progesterone levels, or a combination of both (American Society for Reproductive Medicine and the Society for Reproductive Endocrinology and Infertility, 2021; De Souza, 2003; De Souza et al., 2017). Ovulation occurs but as a result of decreased progesterone, implantation of the oocyte is not supported (De Souza et al., 1998b). There are multiple hypotheses on the etiology of LPD. One proposed mechanism relates to a defective corpus luteum, which is unable to secrete adequate amounts of progesterone during the luteal phase (Boutzios et al., 2013). An alternate mechanism is increased LH pulsatile secretion in the early follicular phase that downregulates the midcycle LH surge, resulting in lower progesterone levels in the luteal phase (American Society for Reproductive Medicine and the Society for Reproductive Endocrinology and Infertility, 2021; Ayabe et al., 1994; Bukulmez & Arici, 2004; J. Jordan et al., 1994; Schliep et al., 2014; Soules et al., 1989; Suh & Betz, 1993).

No widely accepted progesterone cutoff values for LPD have been established for blood samples (American Society for Reproductive Medicine and the Society for Reproductive Endocrinology and Infertility, 2021; Bukulmez & Arici, 2004; De Souza, 2003; Janse De Jonge et al., 2019; Mesen & Young, 2015). In healthy subjects, progesterone responds to LH pulses and can fluctuation from 5-15 ng/mL in a 24-hour time period, indicating that a single value is not accurate to diagnose LPD (American Society for Reproductive Medicine and the Society for Reproductive Endocrinology and Infertility, 2021; Filicori et al., 1984; J. Jordan et al., 1994; Mesen & Young, 2015; Sonntag & Ludwig, 2012). Recommendations of either 1) three daily progesterone values during midluteal phase days 5-9 with a total progesterone <30 ng/ml (J.

Jordan et al., 1994), 2) <5 ng/ml for 5 or more luteal phase days (Schliep et al., 2014), or 3) a single midluteal progesterone measurement <10 ng/ml have been suggested (J. Jordan et al., 1994; Schliep et al., 2014). Another recommendation is to measure urinary pregnanediol glucuronide (PdG), a progesterone metabolite, daily in the first urine void each day in the luteal phase. By measuring the metabolite, it eliminates the variability of the progesterone secretion (Bukulmez & Arici, 2004; Chatterton et al., 1982; Mesen & Young, 2015). It is recommended to use the criteria of 1) the sum of the 3-day midluteal PdG peak of <10 µg/ml *and* 2) a peak PdG concentration <5 µg/ml to define LPD when using urine specimens (De Souza et al., 1998a, 2010a; Santaro et al., 2002). Currently, there are no accepted guidelines or cut points related to LPD for salivary assessments of progesterone and this has not been addressed in the literature.

In LPD, conflicting data of the hormonal profiles of LH, FSH, and E2 are reported and could potentially reflect that most studies only measure a single menstrual cycle with minimal hormone sampling. De Souza et al. is the only investigator to examine LPD in three consecutive menstrual cycles with daily hormone measures in exercising females (De Souza et al., 1997, 1998b, 2004, 2010a). LPD cycles had a longer follicular phase, later ovulation, a shorter luteal phase and decreased PdG compared to ovulatory cycles. FSH was decreased in the last five days of the menstrual cycle, which may explain why decreased follicular estrogen conjugate (E1C) was found. De Souza et al. had conflicting results when reporting LH levels, indicating no difference (De Souza et al., 1998b, 2003) in two studies but showed a decreased LH peak in two additional studies (De Souza et al., 1997, 2010a). Other research has presented decreased LH pulsatility with LPD in exercising females (Loucks et al., 1989). LPD can disturb the subsequent menstrual cycle, which could account for the variation in E2 and is an indication that multiple menstrual cycles should be measured (Liu et al., 2004).

When only one or two menstrual cycles were evaluated, LPD hormonal profiles varied from De Souza's findings. Schliep et al. (2014) also found decreased FSH but in contrast identified decreased E2 in both the follicular and luteal phases over two menstrual cycles (Schliep et al., 2014). Conversely, Soules et al. (1989) and Pirke et al. (1990) investigated one menstrual cycle and discovered lowered E2 concentrations in the luteal phase only (Pirke et al., 1990; Soules et al., 1989) and normal FSH levels (Soules et al., 1989). Other research found neither E2 (Suh & Betz, 1993) or FSH (Grunfeld et al., 1989; Suh & Betz, 1993) to be abnormal. Prior et al. (1990) looked at two nonconsecutive menstrual cycles (month 1 and 12) and identified no difference in E2 or progesterone with shortened luteal phases compared to sedentary controls. However, only one sample was obtained in each phase of the two menstrual cycles (Prior et al., 1990).

LPD occurs in 3-20% of all women but may affect up to 48% of menstrual cycles in exercising females. LPD is the most common menstrual dysfunction in an active population (De Souza, 2003) and has been associated with a hypometabolic state (De Souza et al., 2010a). Yet research in LPD in regularly exercising females is lacking, with most research investigating LPD in untrained females initiating an intense exercise program (Beitins et al., 1991; Bullen et al., 1985).

Anovulation

Anovulation is a failure of the oocyte to expel from the ovary and is associated with low FSH, LH, E2, and progesterone levels (Balasch & Fábregues, 2006; Hamilton-Fairley, 2003; Li & Ng, 2012). Decreased FSH affects development of the ovarian follicles and E2 production, while decreased LH fails to stimulate follicular rupture and subsequent ovulation. Furthermore, failure of luteinization results in decreased progesterone and E2 production (Hamilton-Fairley, 2003; Levi-Setti et al., 2004; Wallach et al., 1995). Menstrual cycle length can remain normal,

extended (i.e., oligomenorrhea), or the menstrual cycle may be absent (i.e., amenorrhea) (De Souza & Williams, 2004).

Ultrasounds are the gold standard to determine follicular growth and rupture but are costly and not widely available (McConnell et al., 2002). Therefore, home urine tests are commonly used as a validated and reliable way to determine ovulation (Gudgeon et al., 1990; Guermandi et al., 2001; Leiva et al., 2014). However, LH surge levels vary in amplitude and duration and there is no consensus on the LH threshold to determine anovulation (Balasch & Fábregues, 2002; Johnson et al., 2015; Park et al., 2007). Thus, it is recommended to confirm ovulation with secondary measures (Janse De Jonge et al., 2019; McGovern et al., 2004) although the literature also disagrees on the necessary progesterone level to confirm ovulation in the midluteal phase. Progesterone levels of 2-3 ng/mL in the mid-luteal phase are indicative of ovulation (American Society for Reproductive Medicine and the Society for Reproductive Endocrinology and Infertility, 2021; Israel et al., 1972; Schliep et al., 2014; Wideman et al., 2013) yet other literature states that 4.5-5 ng/ml (de Jonge, 2003; Janse De Jonge et al., 2019; Landgren et al., 1980) and even 9.4 ng/ml of progesterone (Hull et al., 1982; Li & Ng, 2012) is necessary. Urinary metabolites may also be used to assess ovulation with luteal estrone-1-glucuronide (EIG) peak levels above 35 ng/ml and peak PdG above 5 µg/ml (De Souza et al., 2008; Kesner et al., 1992; Santaro et al., 2002). Again, these criterion cut points have not been established in salivary samples.

Exercising females experience higher anovulation rates (10-58%) (Prior et al., 2015; Wideman et al., 2013) than sedentary females (2-9%) (Johnson et al., 2015). Prior et al. (2015) reported a 58% rate of anovulation, using a progesterone threshold of ≥3 ng/ml for ovulation confirmation across one menstrual cycle in a combined population of sedentary and exercising females with no LH surge confirmation. Whereas Wideman et al. (2013) measured anovulation

in exercising females with a 2 ng/ml progesterone cutoff, multiple sampling days and a LH surge confirmation over two menstrual cycles and reported a 10% prevalence rate. The latter is more common amongst the literature that ranges from 12-21% with LH confirmation and multiple sampling days of progesterone (in serum or urine) over more than one menstrual cycle (De Souza et al., 1998b, 2010b).

Oligomenorrhea

Oligomenorrhea is commonly characterized as irregular and inconsistent menstrual cycles 36-90 days in length (De Souza & Williams, 2005; Loucks & Horvath, 1984; Riaz & Parekh, 2022) or 4-9 menstrual cycles per year (Barrack et al., 2014; Gibbs et al., 2014). Oligomenorrhea is similar to amenorrhea but presents as an insufficient stimulation, not a complete suppression, of the hypothalamus-pituitary-gonadal (HPG) axis (Baggio et al., 2019). Ovulation may or may not occur (De Souza et al., 2010b) and severe oligomenorrhea can present with decreased LH pulse frequency (Veldhuis et al., 1985). E2 is typically low due to the follicles struggling to achieve dominance but is also erratic with E2 being produced independent of ovulation. Hormone levels are rarely assessed in oligomenorrheic exercising females but Reed et al. (2015) reported erratic E1G concentrations in 30 oligomenorrheic women across a 28-day period (Reed et al., 2015).

It is hard to determine the prevalence of oligomenorrhea in a physically active population, due to the fact that oligomenorrhea and amenorrhea results are commonly combined (Cokkinades et al., 1990; Koltun et al., 2020; Rosetta et al., 2001; Rosetta, Harrison, et al., 1998; Rosetta, Williams, et al., 1998), or the data is a combined population (e.g., elite and recreational) (Barrack et al., 2014; Burrows, 2003; Gibbs et al., 2013). Oligomenorrhea prevalence in all athletic populations (i.e., elite, recreational) ranges from 0.9%-52.5% (Gibbs et al., 2013), with a lower prevalence in recreationally active females, ranging from 6.6-18%

(Folscher et al., 2015; Shangold & Levine, 1982). Oligomenorrhea was reported in 6.6% of ultra-marathon runners (Folscher et al., 2015), while Shangold et al. (1982) observed 18% of marathon runners had oligomenorrhea. Prevalence of oligomenorrhea is commonly assessed with unvalidated questionnaires (Cokkinades et al., 1990; Gray & Dale, 1983; Hetland et al., 1995; Reed et al., 2015; Rosetta et al., 2001; Rosetta, Harrison, et al., 1998, 1998; Shangold & Levine, 1982; Thompson & Gabriel, 2004) with only two studies determining oligomenorrhea with a validated questionnaire (i.e., Low Energy Availability in Females Questionnaire (LEAF-Q)) (Folscher et al., 2015; Meng et al., 2020).

Secondary Amenorrhea

Amenorrhea is the most severe reproductive dysfunction that presents as chronic anovulation and is defined as the absence of three or more consecutive menstrual cycles (Allaway et al., 2016; American Academy of Pediatrics et al., 2006; De Souza & Williams, 2005; Klein et al., 2019; Roupas & Georgopoulos, 2011). Some literature applies more stringent criteria (i.e., absence of 6 or more menstrual cycles) but the former is recommended due to the high risk of health complications (e.g., decreased bone mineral density) associated with amenorrhea (American Academy of Pediatrics et al., 2006; De Souza & Williams, 2005). Functional hypothalamic amenorrhea (FHA) is a form of secondary amenorrhea (i.e., cessation of the menstrual cycle after menarche) and is not due to identifiable organic causes (e.g., polycystic ovarian syndrome) but can be corrected by changing behavioral factors such as exercise, energy availability, and stress (Gordon et al., 2017). An energy deficit is a common trigger that disrupts GnRH secretion and results in diminished LH, FSH, E2, and progesterone levels (Gordon et al., 2017; Klein et al., 2019), that do not contain the normal hormonal peaks seen across the normal menstrual cycle (Allaway et al., 2016). A reduction in FSH can decrease E2 while a decrease in LH will disrupt progesterone secreted by the corpus luteum (Allaway et

al., 2016; De Souza et al., 1998b, 2010b). LH presents with decreased amplitude or erratic pulses that resemble LH in early puberty (Allaway et al., 2016; Loucks et al., 1989; Veldhuis et al., 1985). FSH pulsatility has not been well characterized in amenorrhea (Loucks & Thuma, 2003) but Fisher et al. (1986) found no difference in LH or FSH pulse frequency with amenorrhea (Fisher et al., 1986).

The prevalence of amenorrhea ranges from 2-5% in sedentary females (Allaway et al., 2016; De Souza & Williams, 2005), 1-60% in all physically active females (i.e., recreational, elite) (Gibbs et al., 2013), and 0-31% in recreationally active females (Boyden et al., 1984; Rosetta, Williams, et al., 1998; Thompson & Gabriel, 2004). However, many exercising females have low knowledge levels about whether or not oligomenorrhea and amenorrhea are abnormal, therefore it could be underreported (Folscher et al., 2015). Even though amenorrhea is a severe condition, it is reversable. Menses was successfully resumed in amenorrheic athletes by adding only 330-360 kcal/day for twelve and six months respectively (Cialdella-Kam et al., 2014; De Souza et al., 2021).

Measures of Sex Steroid Hormones

Hormones measured through serum are costly, invasive, and have a high participant burden when multiple timepoints across the menstrual cycle need to be measured. Urine and saliva are alternate methods to measure hormone concentrations and allows the participant to collect the specimen at home, allowing a lower participant burden and potentially higher participant retention when daily sampling is required. Urine and saliva are both stable at room temperature for a few days, considered non biohazardous, and are easily obtained. However, each method measures hormone concentrations differently. While serum measures hormones at a single point in time, urine represents a pooled value of hormone concentrations over 6-8 hours and saliva represents levels over a few hours. Additionally, urine only has metabolites of

E2 and progestogen and have variable water content that require additional measurements (i.e., creatinine for correction), while saliva does not require any additional steps. Measurements of E2 and progesterone in saliva and urine also have strong correlations with serum concentrations in premenopausal women (Bellem et al., 2011).

Saliva's correlation with serum is strongest in women of reproductive age and decreases when E2 and progesterone are low in populations such as postmenopausal women and prepubescent children (Bellem et al., 2011; Shirtcliff et al., 2000). Albeit serum hormone concentrations are the gold standard, saliva can be a useful tool in diagnosing menstrual dysfunction but further research is needed as no cutoff points for menstrual dysfunction have been well established. Ishiwaka et al. (2002) compared serum and saliva in 121 women and determined a saliva cutoff value of 189 pmol/L (51.48 pg/mL) in the midluteal phase provided high diagnostic efficiency for diagnosing luteal phase defect (LPD) (Ishikawa et al., 2002). Progesterone levels of 60 pg/mL or greater are indicative of ovulation when compared to serum threshold of 4 ng/mL (Codner, Eyzaguirre, et al., 2011; Codner, Villarroel, et al., 2011; Gandara et al., 2007). However, a secondary analysis of seven studies that measured salivary E2 and progesterone concentrations cautioned against using immunoassays to analyze salivary E2 and progesterone and found the highest validity with mass spectrometry. A weak association was found between estradiol and menstrual cycle phase, potentially due to the small concentrations of E2 available in saliva and low sensitivity of immunoassays. Progesterone had a higher association than E2 with menstrual cycle phase but demonstrated variability based on the assay used. Nonetheless, all the research used in this secondary analysis did not always control for the menstrual cycle and in some cases, only took two measures (Arslan et al., 2022).

Energy Availability and Menstrual Dysfunction

The relationship between LEA and menstrual function in exercising women is confirmed in both short term (Loucks & Thuma, 2003) and long-term studies (Williams et al., 2015). The exact mechanism of LEA in menstrual disorders has yet to be determined but LEA is known to disrupt GnRH and LH. Decreased LH pulsatility occurred in only five days with sedentary females restricted to an EA below 30kcal/kg/FFM/day (Loucks & Thuma, 2003). An energy availability threshold of 30 kcal/kg/FFM/day was originally thought to induce menstrual dysfunction but more recent research has indicated otherwise (De Souza et al., 2019). In a cross-sectional study, the 30 kcal/kg/FFM/day threshold discriminated amenorrhea from eumenorrhea but not from subclinical menstrual disorders (Reed et al., 2015). Additionally, a three-month diet and exercise intervention in eumenorrheic sedentary females induced menstrual dysfunction that occurred above the 30 kcal/kg/FFM/day threshold and alternatively menstrual function remained normal in some females that were below the 30 kcal/kg/FFM/day threshold (Williams et al., 2015). Even though the threshold was unsupported, a linear relationship did emerge for energy availability and menstrual dysfunction risk, identifying a 50% risk of menstrual dysfunction occurring if energy availability is below 30 kcal/kg/FFM/day (Lieberman et al., 2018). Thus, these findings indicate that a greater understanding of the relationship between menstrual cycle hormones and energy availability is needed. Additionally, this formula has limitations due to total energy expenditure is calculated by more than exercise (i.e., resting metabolic rate, thermic effect of food, physical activity) but was created due to the complexity of measuring all the components of energy availability.

The etiology of LEA is likely specific to the individual and may be intentional or unintentional. Energy intake may be reduced intentionally to decrease body fat percentage, to optimize performance or for social reasons, with many young females attempting to lose weight

to improve appearance (Martinsen et al., 2010). Eating disorders (clinical and subclinical) are another potential cause and are hard to distinguish from athletes who are only trying to improve performance and should be evaluated by a medical professional. LEA may unintentionally be caused by suppression of appetite by prolonged exercise, as exercise stimulates anorexigenic hormones (Hackney & Constantini, 2020) or by consuming a low energy density diet (Melin et al., 2016). Regardless of the origin, LEA must be identified and corrected before detrimental long term effects (i.e., bone mineral density loss, infertility) materialize (De Souza et al., 2017; Mountjoy et al., 2014).

Nonetheless, daily or average LEA are not the only factors to consider with menstrual dysfunction and warrants a more in-depth analyses of LEA. Fahrenholtz et al. (2018) measured energy availability every hour for 24 hours in elite athletes and revealed that there was no difference in energy availability between the eumenorrheic and menstrual dysfunction (i.e., oligomenorrhea, amenorrhea) groups. Yet, the menstrual dysfunction group had decreased E2 and resting metabolic rate (RMR) ratio (i.e., measured RMR: predicted RMR) but importantly, the menstrual dysfunction group also experienced a greater within-day energy deficiency, spending four hours more in an energy deficient state (i.e., -300kcal/hour/day) than the eumenorrheic group (Fahrenholtz et al., 2018).

Sex Steroid Hormone Influences on Energy Availability

Energy intake can vary between 100-300 kcal across the menstrual cycle but there is no consensus regarding fluctuations in macronutrient intake across the cycle (Buffenstein et al., 1995; Chappell & Hackney, 1997; Hirschberg, 2012). Energy intake is the lowest in the follicular phase and peaks in the luteal phase (Barr et al., 1995; Buffenstein et al., 1995; Rocha-Rodrigues et al., 2021), potentially due to the energetic costs of reproduction (Jasienska, 2003) and sex hormones modulating energy intake (Asarian & Geary, 2006). Menstrual function may

also influence energy intake as women with anovulatory cycles (i.e., decreased estrogen and progesterone) had unchanged energy intake across the menstrual cycle (Barr et al., 1995; Rock et al., 1996) further indicating that research should look at the associations between menstrual dysfunction and energy intake.

E2 and progesterone directly and indirectly affect energy intake. E2 is considered a potential appetite suppressant but the role of progesterone is not fully elucidated. Progesterone by itself does not change eating behavior in rats but stimulates appetite when E2 is present (Hirschberg, 2012). Energy intake decreases when E2 is high, with energy intake being the lowest prior to ovulation, which is when E2 is the highest, and can result in an ~10% decrease in energy intake (Asarian & Geary, 2006). Additionally, postmenopausal women and women with ovariectomies demonstrated increased energy intake but E2 replacement therapy normalized energy intake (López & Tena-Sempere, 2015).

E2 acts centrally on the hypothalamus and alters neural processing of feedback signals that control eating. Mice with deleted brain estrogen receptor (ER) α experienced increased abdominal obesity that resulted from hyperphagia (i.e., increased appetite) and hypometabolism (Xu et al., 2011). Additionally, an association between the Erα receptors and anorexia nervosa was found, indicating a potential role of E2 and restricted eating (Versini et al., 2010). E2 may exhibit a bidirectional role on energy intake depending on the energy state. Previous research has indicated that when mice are in a satiated state, E2 inhibits feeding (Dragano et al., 2020). When ovariectomized (OVX) mice (i.e., no endogenous E2) were treated with E2, hyperphagia occurred. Interestingly, when OVX mice were fasted overnight, refeeding was slower than the control mice but when treated with E2 refeeding was the same (Yu et al., 2020). This indicated that E2 is required to adapt to refeeding from an energy deficient state, which may be an important factor for anorexic and postmenopausal women that are in an E2-deprived state.

Resting Metabolic Rate (RMR), Energy Availability and Menstrual Function

RMR is measured via indirect calorimetry and can be calculated from FFM (e.g., Cunningham equation) or dual energy X-ray absorptiometry (DXA) but only DXA considers all metabolically active tissues. RMR can be an additional method to determine LEA, with values 20-40% less than expected in chronically energy-deficient women (Jasienska, 2003). The ratio of measured (i.e., indirect calorimetry) to predicted RMR (i.e., Cunningham, DXA) (mRMR:pRMR) is correlated with total triiodothyronine (TT_3) levels, which is a biomarker of energy deficiency. RMR ratio cutoff values below .90 and .94 for the Cunningham equation and DXA respectively, are indicators of LEA and have proven to be reliable measures regardless of menstrual function (i.e., amenorrhea, LPD) (Strock, Koltun, Mallinson, et al., 2020; Strock, Koltun, Southmayd, et al., 2020).

When using indirect calorimetry and DXA-predicted RMR, ovulatory women displayed similar measured and predicted RMR. In contrast, amenorrheic females had ~8% lower measured RMR than DXA-predicted RMR and had higher predicted RMR than the ovulatory controls. FFM and fat mass (FM) were similar in both groups but the amenorrheic group had 13% higher residual mass (e.g., organ tissue, soft tissues). Given that the amenorrheic group had lower RMR but not reduced tissues, this is an indicator that amenorrhea is not caused from decreased tissue mass but potentially metabolic and endocrine adaptations at the tissue level that are indicative of energy conservation (Koehler et al., 2016). Conversely, DXA-predicted RMR was similar between ovulatory, menstrual dysfunction (i.e., oligomenorrhea, LPD, anovulation), and amenorrheic groups but the metabolically active body compartments were not reported so it is unclear if there was a difference in residual mass as in the Koehler et al. study. Furthermore, indirect calorimetry RMR and the RMR ratio with DXA predicted RMR was significantly lower in all amenorrheic groups compared to subclinical menstrual dysfunction

groups and ovulating controls (De Souza et al., 2007; Strock, Koltun, Southmayd, et al., 2020). All groups had similar FFM but the ovulatory and the subclinical menstrual dysfunction group had significantly higher body fat percentage, body mass, and TT_3 than the amenorrheic group. RMR is commonly accepted as being lower in amenorrheic exercising females when compared to eumenorrheic (De Souza & Williams, 2005; Myerson et al., 1991) yet Koltun et al. (2020) reported oligomenorrheic and amenorrheic females as having similar RMR and TT_3 compared to eumenorrheic females (Koltun et al., 2020). The oligomenorrheic and amenorrheic females were reported as a single group and were classified by medical history. However, an average menstrual cycle length of 32 days was reported, which may indicate that even though the oligomenorrheic/amenorrheic group had decreased E1G and PdG compared to the eumenorrheic females, an energy deficit may be present but not be severe enough during data collection to result in a decreased RMR. Furthermore, when amenorrhoeics increased energy intake and maintained consistent exercise volume for 6 months resulting in increased energy availability, menses resumed but RMR did not change with the intervention. RMR was similar to eumenorrheic controls and when expressed in relative terms, RMR was actually higher in the amenorrheic group. The amenorrheic group exercised an average of 250 minutes more per week compared to the control group, which is consistent with previous research that indicated higher volume or frequency of physically activity may result in higher RMR due to the residual effect of exercise on RMR (Bullough et al., 1995). Additionally, both groups displayed similar body fat percentage and FFM prior to the intervention and after 6 months, FFM did not change but body fat percentage did increase in the amenorrheic group (Guebels et al., 2014).

A recent meta-analysis was unable to determine the influence of the menstrual cycle on RMR due to most research not controlling for the phase of the menstrual cycle (Benton et al., 2020). Therefore, it is indiscernible if RMR fluctuates with the menstrual cycle, with some

research indicating it is stable (Elliott et al., 2015; Howe et al., 1993) while other research has described variability across the menstrual cycle (Day et al., 2005; Henry et al., 2003). Thus, further research is needed with RMR and the menstrual cycle that incorporates control for menstrual cycle phase and hormonal confirmation of menstrual cycle dysfunction.

Body Composition

Menstrual dysfunction was originally thought to be caused by low body fat percentage. This hypothesis is no longer accepted, given that research could not consistently verify an association of menstrual status with body composition (Loucks & Horvath, 1985). There are low body fat eumenorrheic females (Baker et al., 1981) and normal to high body fat amenorrheic females (McArthur et al., 1980), implicating that there is no critical body fat threshold to maintain menstrual function (Loucks, 2003; Loucks & Horvath, 1985). Furthermore, when female rats were kept underweight, puberty was delayed. In less than 24 hours after ad libitum feeding, LH pulsatility began and occurred before any weight could be regained, demonstrating a coupling of energy intake and the GnRH pulse generator (Bronson, 1986)—although this study used a rodent model, it indicates that further research is needed to determine if similar results would be seen in humans.

Adipose tissue is a conversion site for androgens to estrogens, indicating a potential correlation of body fat percentage and estrogens (Loucks & Horvath, 1985). Conversely, a U-shaped association with E2 to body fat was discovered in exercising eumenorrheic females across the menstrual cycle, with both low body fat percentages (≤22%) and high body fat percentage (>30.8%) resulting in decreased E2 concentrations in women (Ziomkiewicz et al., 2008).

Leptin is secreted from adipose tissue and therefore has been investigated as a link between the adipocyte and reproductive system (Thong et al., 2007). Leptin concentrations are

correlated to adiposity and relays nutritional status to the hypothalamus (Corr et al., 2011), such that low leptin and disruption of the diurnal rhythm is also associated with menstrual dysfunction and LEA (De Souza et al., 2003). Changes in leptin levels have been observed with increased energy intake after energy restriction, with the increase in leptin occurring prior to changes in adiposity. Therefore, changes in leptin levels are believed to be dependent on energy intake and energy availability (Hilton & Loucks, 2000; Loucks, 2003).

Diet

LEA does not always result in menstrual dysfunction; therefore, energy intake should be evaluated separately from energy availability and in detail. Additionally, previous research has identified exercising eumenorrheic and amenorrheic females with similar total energy intake (Broocks et al., 1990; Burrows, 2003; Friday & Drinkwater, 1993; Laughlin & Yen, 1996; Perry et al., 1996; Petkus et al., 2019; Rosetta et al., 2001; Rosetta, Williams, et al., 1998) and similar macronutrients (Perry et al., 1996; Rosetta et al., 2001; Rosetta, Williams, et al., 1998) with only a few studies reporting lower fat intake (Friday & Drinkwater, 1993; Laughlin & Yen, 1996). Furthermore, no difference was found between LPD, anovulatory cycles and controls in energy intake but information on macronutrients was not provided (Broocks et al., 1990; Klein et al., 2019; Reed et al., 2015).

Most research focuses on energy intake and energy availability with few studies investigating diets and nutrient intake associated with menstrual function. The BioCycle study investigated 259 eumenorrheic females over two menstrual cycles and performed 24-hour dietary recalls 4 times across each menstrual cycle to investigate the effects of diet on ovulation. Decreased E2 levels were associated with low vitamin D and increased dairy and riboflavin intake (Harmon et al., 2020; K. Kim et al., 2017, 2020). Moderate amounts of caffeine (i.e., ≥200mg/d) was inversely related with E2 in Caucasian women only, yet caffeinated soda,

green tea intake, and high added sugar and fructose beverages were positively associated with increased E2 in all races (Schliep et al., 2012, 2013).

High fiber intake was inversely associated with E2, progesterone, LH, and FSH and positively associated with risk of anovulation (Gaskins et al., 2009) and LPD (Andrews et al., 2015). This research agrees with previous studies that also reported a relationship between high fiber intake and decreased E2 and progesterone (Barr et al., 1995; Boyd et al., 1997). The effect of high fiber intake on LH levels was attenuated after factoring in E2 levels, indicating that fiber may impact LH through decreased E2, not LH directly (Gaskins et al., 2012). In addition to high fiber, LPD was associated with high isoflavone and low selenium intake (Andrews et al., 2015). Chavarro et al. (2007) found a 'fertility diet' (i.e., high in monosaturated fats, vegetable protein, iron, high-fat dairy) decreased anovulation (Chavarro et al., 2007). The Biocycle study did not find any 'fertility diet' associations when analyzed with LPD cycles, indicating that LPD and anovulation might be affected differently by dietary components (Andrews et al., 2015). While both studies included exercising participants, it was not an inclusion criterion for either study and both studies reported a lower anovulation and LPD rate than is typically reported in exercising females.

Special diets are increasing amongst athletes and need to be examined with menstrual dysfunction. 62% of runners consumed a 'special diet' compared to 13% of controls, with vegan/vegetarian being the most popular (12%). The Mediterranean diet was associated with increased LPD even though this diet is linked to many other health benefits (Andrews et al., 2015). However, the Mediterranean diet is typically high in fruits and vegetables which are low in energy density and that could result in unintentional LEA (Witkoś & Hartman-Petrycka, 2022).

Calculating energy intake through food logs produces a large burden on the participant and researchers, results are prone to underreporting and food logs are known to change usual

intake, which may not give an accurate representation of the long term intake (Burke et al., 2018). A meta-analysis determined that self-reporting energy intake resulted in 19% underreporting of energy intake in athletes, resulting in an average underestimate of 600 kcal (Capling et al., 2017). A 600 kcal deficit could inappropriately categorize an athlete as LEA so to minimize underreporting, it is recommended that food logs should be validated using Goldberg or Black cutoffs (A. Black, 2000; Goldberg et al., 1991). The Goldberg method assumes energy intake = energy expenditure. A cut-off value based off the ratio of reported energy intake to basal metabolic rate (BMR) is used but varies from .9-1.28 depending on the researcher but this method could exclude someone with low energy availability. However, an actual measurement of energy expenditure through an accelerometer or heart rate would allow energy intake to be compared directly to energy expenditure and the Goldberg cutoff would no longer be needed (A. Black, 2000; A. E. Black, 2000).

An alternative method to traditional paper food logs is mobile dietary apps. Mobile apps link directly to databases therefore no data entry by the study personal is required. Additionally, mobile dietary apps allow real time recording due to the portability of the app and features such as bar code scanning and image taking. The ease of the mobile app can decrease subject burden and improve reporting (Pendergast et al., 2017). MyFitnessPal is a validated smartphone app and with reliable dietary analysis (Evenepoel, Clevers, Deroover, Loo, et al., 2020; Evenepoel, Clevers, Deroover, Matthys, et al., 2020; Teixeira et al., 2018). Furthermore, MyFitnessPal is a popular commercial app with over 165 million users in 2016 and is the preferred app of sport dietitians in multiple countries. The MyFitnessPal data base is extensive, with over 6 million food items and brands (Evenepoel, Clevers, Deroover, Loo, et al., 2020).

Low Energy Availability Assessment with Questionnaires

Questionnaires are a less time consuming and less costly method of determining energy availability and can act as surrogate markers to determine LEA risk. However, questionnaires may not properly identify athletes that unintentionally fail to increase energy intake with increased energy demands (Sim & Burns, 2021). The Low Energy Availability in Females-Questionnaire (LEAF-Q) evaluates LEA by assessing symptoms associated with LEA (i.e., injury, menstrual and gastrointestinal function). The LEAF-Q demonstrates a high prevalence of LEA in recreationally active females, ranging from 16-63% (K. Black et al., 2018; Sharps et al., 2021) with the most common prevalence ranging from 35-45% (Folscher et al., 2015; Logue et al., 2019; Meng et al., 2020; Slater et al., 2016). However, the LEAF-Q only evaluates LEA symptoms and may not be appropriate for preventative measures, as the LEAF-Q may be unable to appropriately identify exercising females at high risk that only manifest with subclinical symptoms. Furthermore, it does not assess eating behavior or exercise. Therefore, the LEAF-Q should be used as a screening tool, not as a diagnostic tool (Rogers et al., 2021).

Physical Activity and Exercise

Both multiple weeks of high intensity (Bullen et al., 1985) and an average exercise volume of over 60 minutes per day (Green et al., 1986) are known to increase risk of menstrual dysfunction. Exercise may not be the primary stressor for menstrual dysfunction although the energy cost of exercise does influence energy availability (Loucks, 2003). Loucks et al. (2003) performed an intervention with exercise and LEA and discovered LH pulsatility was disrupted only in the females with LEA, not with exercise alone (Loucks & Thuma, 2003). Williams et al. (2001) furthered this hypothesis by inducing amenorrhea in monkeys through an exercise and restricted diet intervention. When energy intake was increased, normal menses resumed even when exercise remained constant (Williams et al., 2001).

Only two studies to date have investigated exercise energy expenditure, energy intake, and subclinical menstrual dysfunction. Reed et al. (2015) examined exercising ovulatory, inconsistent ovulatory, oligomenorrhea, and amenorrheic females with similar EEE (i.e., no difference in exercise volume, frequency, or intensity), energy intake and energy availability. The RMR ratio (mRMR:pRMR) differentiated the oligomenorrhea and amenorrhea groups when compared to ovulatory and subclinical menstrual dysfunction (Reed et al., 2015). De Souza et al. (1998) examined exercising ovulatory, LPD, and anovulatory groups. LPD and ovulatory groups were similar in EEE, energy intake, energy availability, and macronutrients. However, anovulatory had a higher 24-hour energy expenditure and EEE with lower energy availability, carbohydrate intake, and fat intake when compared to LPD (De Souza et al., 1998b).

Energy intake may be similar regardless of menstrual function, therefore EEE needs to be examined closely to accurately determine energy availability. Yet, guidelines to calculate energy availability are unclear, both in length of assessment and methods used making it difficult to accurately assess these variables (Burke et al., 2018). The most common method to estimate EEE is through Metabolic Equivalent of Task (METS) from activity logs or questionnaires but these do not directly measure EEE (Burke et al., 2018). Ainsworth et al. (2000) derived the compendium of physical activity that can be used to determine the appropriate MET level for the exercise performed (Ainsworth et al., 2000).

Accelerometers are used to estimate energy expenditure and are commonly wrist or hip worn. Common issues with accelerometers are decreased participant comfort and compliance, along with accelerometers underestimating activity in free living conditions (O'Driscoll et al., 2020). When compared to doubly labeled water, some accelerometers underestimated between 100-600 kcal, depending on the device (Murakami et al., 2016, 2019). Total physical activity energy expenditure can be assessed with accelerometers but varies greatly depending on the

device and daily activities (e.g., running, household tasks), with energy expenditure being underestimated in many devices. Pairing accelerometers with heart rate can improve the estimates of energy expenditure but still presents limitations with non-weight-bearing activities (e.g., cycling). Conversely, this pairing tends to moderately overestimate energy expenditure during ambulation and stair climbing (O'Driscoll et al., 2020).

EEE can also be measured via a heart rate monitor and the heart rate index method ($6*(HR_{absolute}/HR_{rhr})-5$) that determines the metabolic equivalent of task (MET). The heart rate index is a validated measure that has proven to be an accurate measure of VO_2 and different intensities (KANG et al., 2020; Wicks et al., 2011; Wicks & Oldridge, 2016). A MET is equal to a resting value of 3.5 ml/kg/min but this varies by participant and females typically have lower values. Therefore, corrected METs are recommended to avoid over or underestimation of EEE. Corrected METs can be calculated by using a correction factor by dividing 3.5ml/kg/min by indirect calorimeter VO_2 ml/kg/min (Correct MET value = MET value * 3.5ml/kg/min / VO_2 ml/kg/min (Ainsworth et al., 2011; Byrne et al., 2005; Kozey et al., 2010).

The definition of exercise is inconsistent, with some research using only purposeful exercise, while others include leisure activities or other activities at a lower intensity level than prescribed (Burke et al., 2018). Guebels et al. (2014) demonstrated that the calculation of energy availability is highly dependent on the methods chosen. Energy intake was assessed with a 7-day food log and EEE was calculated from activity logs and accelerometers. Four exercise energy expenditure methods were used to determine energy availability 1) all planned exercise 2) all planned exercise plus bike commuting and all walking 3) all exercise at 4 METS and greater (≥ 4.0 METS) and 4) all exercise greater than 4 METS (> 4.0 METS). Bicycle commuting was the only activity equal to 4 METS and was the only difference between method 3 and 4. A 30% variation in energy availability resulted, depending on the method chosen.

When energy availability was expressed in relative terms, the values ranged from 28.2-36.7 kcal/kg/FFM/d, falling above and below the previously proposed threshold of 30 kcal/kg/FFM/d for LEA. All planned exercise plus bike commuting and walking gave the largest EEE, and all exercise > 4.0 METS gave the smallest. Method 1 and 3 were comparable, with a difference ranging from ~10-87 kcal/d (Guebels et al., 2014).

Most research participants are endurance athletes or 'leanness' sports (e.g. ballet, gymnastics), which have been found to have a high prevalence of LEA and menstrual dysfunction (Roupas & Georgopoulos, 2011). However, less is known about the risk levels in females that are general exercisers, power sports, etc. and deserves further investigation. Classification of activity level can be subjective when using terms such as 'trained' or 'elite' athlete. Even with 'recreational' athletes, this term normally refers to an active individual that is not a professional or collegiate athlete, but total exercise can vary greatly. McKay et al. (2022) has proposed a five-tier system that would help classify individuals from sedentary to world class. Tier 4 and 5 classify the high-performance athletes as world class/Olympic level (Tier 5) or competing internationally along with National Collegiate Athletic Association (NCAA) Division I athletes as Tier 4. Tier 3 consists of athletes that are training at near maximal training volume for their respective sport, compete at the national level, or are NCAA Division II or III athletes. Athletes that are training 3 times or more per week with the intent to compete are considered trained (Tier 2) while recreationally active athletes (Tier 1) must meet the World Health Organization (WHO) 2020 guidelines for physical activity which are to perform per week either two sessions of muscle-strengthening activities, 150 minutes of moderate activity, 75 minutes of vigorous activity, or an equivalent combination of moderate and vigorous activity (Bull et al., 2020). Anyone that does not meet the WHO guidelines is classified as sedentary (Tier 0) (McKay et al., 2022). Additionally, a flow-chart is supplied to assist in determining the category

however the classification may need to be adjusted per sport, especially with Tier 2 and 3 athletes. In individual sports (e.g., triathlon, cycling) competitive amateur athletes win events while competing against NCAA athletes outside of collegiate competitions, displaying the need for Tier 2 and 3 to be clarified. Also, competitive amateur athletes differ greatly in proficiency and training volume and may need additional tiers. For example, USA cycling categorizes racers from 5 (i.e., novice) to 1 (i.e., elite) but all levels are considered competitive amateurs and would fall under Tier 2. Regardless, this tier system is a much needed first step in classifying athletes and could be beneficial in identifying menstrual dysfunction and LEA within different athletic levels.

Confounding Factors of Menstrual Dysfunction

Stress and Recovery

Regarding menstrual dysfunction, the stress of exercise should be examined. Exercise stress with proper recovery is required to stimulate adaptations from exercise and varies amongst individuals. However, when excessive stress or inadequate recovery occurs, individuals may experience a physiological or psychological imbalance that results in deleterious conditions and decreased performance (Kellmann & Kolling, 2019). Individuals in a functional overreaching, nonfunctional overreaching, and over training state have demonstrated similar symptoms (e.g., decreased E2, progesterone, T_3) to LEA (Stellingwerff et al., 2021), yet energy availability and menstrual function are rarely assessed in these states. Therefore, future research is indicated to examine the relationship between stress-recovery states and energy availability and the menstrual cycle.

Exercise stress can be estimated using algorithms that are designed to calculate training stress, training load, and fatigue from each exercise session. Training Peaks is an online platform that allows exercise to be synced from various devices and apps to allow analyses of

chronic training load (CTL), training stress score (TSS), acute training load (ATL), and training stress balance (TSB). Chronic training load combines duration and intensity to provide a value of how much the athlete has trained historically. TSS is an estimate of the training load based off intensity and duration and can be used to determine how much recovery may be needed after an exercise session. TSS has also demonstrated a strong dose-response relationship for changes in aerobic fitness (Sanders et al., 2017). ATL assess fatigue based off duration and intensity over the past seven days. Finally, TSB represents the balance of training stress, and the value is representative for the following day. A negative TSB indicates that an individual is not adapted to a training load (e.g., fatigued, low form) while a neutral or positive number indicates potentially adapted or over adapted (e.g., 'fresh', high form) to a training load (*TrainingPeaks*, n.d.).

However, external training load alone is not sufficient to determine stress and recovery and the athletes internal load such as ratings of perceived exertion (RPE) and stress-recovery state also needs to be considered. A higher training load (i.e., TSS) is associated with a higher RPE (Alfonso & Capdevila, 2022), further substantiating RPE can be an accurate measure of internal load. Additionally, a high RPE with a low training load could be an indicator of an imbalanced stress-recovery state, indicating a need to report RPE with exercise. Surveys such as the Acute Recovery and Stress Scale (ARSS) use a multidimensional approach by assessing the current recovery-stress state at an emotional, mental, physical, and overall level (Kellmann & Kolling, 2019). The ARSS has been validated in monitoring acute recovery and stress in athletes (Kölling et al., 2015, 2020).

Reproductive Maturity

It has been proposed that age, pregnancy, early age of menarche, training status prior to menarche, and previous menstrual history are factors in current menstrual dysfunction. The

average age of menarche (i.e., onset of menstruation) is between 12-13 years and is affected by racial and ethnic differences (Skinner, 2018). If menarche does not occur by 15 years or three years post-thelarche, it is considered primary amenorrhea (Klein et al., 2019; Roupas & Georgopoulos, 2011; Seppä et al., 2021). A higher prevalence of current menstrual dysfunction is associated with a later age of menarche (Anai et al., 2001; Baker et al., 1981; Feicht et al., 1978; Fisher et al., 1986; Frisch et al., 1980; Koltun et al., 2020; Loucks et al., 1989; Reed et al., 2015) and a lower gynecological age (i.e., number of years since menarche) (Fisher et al., 1986; Koltun et al., 2020; Reed et al., 2015). Conversely, only a small number of studies have not found an association between age of menarche (Laughlin & Yen, 1996; Schwartz et al., 1981; Thompson & Gabriel, 2004) or gynecological age (Laughlin & Yen, 1996) and menstrual function. Age of menarche (Broocks et al., 1990; De Souza et al., 1997, 2004) and gynecological age (Broocks et al., 1990; De Souza et al., 1997; Pirke et al., 1990) appear to have no influence on subclinical dysfunction such as LPD or anovulatory cycles.

Collegiate athletes that started athletic training post menarche achieved menarche three years earlier and experienced less oligomenorrheic and amenorrheic cycles than their peers that started training prior to menarche (Frisch, 1981). In Fisher et al. (1986), all participants began training after menarche, yet amenorrheic females had a later age of menarche and started running at an earlier age that was closer to menarche than the eumenorrheic runners (Fisher et al., 1986). Finnish club athletes from endurance, aesthetic, technical (e.g., horse riding), ball games, and power sports and nonathletes reported menstrual cycles between 14-16 years then again when the athletes were 18-20 years. The biggest predictor of current menstrual dysfunction was past menstrual dysfunction in athletes and nonathletes (Ravi et al., 2021). Menstrual cycles are often irregular through adolescence with 60-80% of menstrual cycles consistently staying between 21-35 days by the third year post menarche (American

Academy of Pediatrics et al., 2006). Since athletes with menstrual dysfunction had a higher age of menarche than eumenorrheic athletes and all menstrual dysfunction athletes were grouped together (i.e., primary and secondary amenorrhea, oligomenorrhea), it is difficult to determine if menstrual dysfunction is due to gynecological age or other factors.

Research suggests that menstrual function prior to the start of athletic training is highly associated with current menstrual irregularity (Cokkinades et al., 1990; Gray & Dale, 1983; Lutter & Cushman, 1982; Schwartz et al., 1981; Shangold & Levine, 1982). Additionally, nulliparous females have a higher prevalence of amenorrhea compared to females who have had at least one pregnancy (Baker et al., 1981; Dale et al., 1979; Loucks et al., 1989; Schwartz et al., 1981). However, age should be considered. The nulliparous females were younger than the parous females and the younger females (i.e., less than 30 years) had a significantly higher amenorrhea rate regardless of pregnancy history (Baker et al., 1981).

Fat Free Mass (FFM)

Body weight and body mass index (BMI) are useful common measures to track overtime but they do not provide insight into body composition. BMI is discouraged as the only tracking measure, as it can be misleading and be inaccurate measure in active populations that have high FFM. FFM is the body's most metabolically active tissues (Mountjoy et al., 2014) and is composed of brain, bone, and skeletal muscle mass. Furthermore, FFM can be used to calculate RMR which is used as a measure of energy status (De Souza et al., 2019) but current research is conflicting as to the effect of FFM on menstrual function. FFM percentage was lower and body fat percentage was higher in exercising females with subclinical menstrual dysfunction than the eumenorrheic exercising females (Broocks et al., 1990) but when investigating only LPD, higher fat mass but no difference in FFM was found (Schaumberg et al., 2017). Reed et al. (2015) determined no difference in FFM when investigating regular, irregular, anovulatory,

oligomenorrheic, and amenorrheic exercising females (Reed et al., 2015) with similar results found in amenorrheic and eumenorrheic females (Koehler et al., 2016; Laughlin & Yen, 1996). However, oligomenorrheic/amenorrheic exercising females with LEA had lower FFM and body fat percentage when compared to eumenorrheic controls (Koltun et al., 2020).

Additionally, sex steroid conversions take place in muscle, indicating a need to assess FFM when assessing the menstrual cycle (Loucks & Horvath, 1985). Estrogen in skeletal muscles can stimulate growth of muscles and can inhibit inflammatory pathways. Decreased estrogen has been associated with decreased FFM but the effects of progesterone are less studied and the effects are unclear (Y. J. Kim et al., 2016).

Conclusion

Menstrual function and energy availability are coupled in exercising females; therefore, both should be evaluated. Additionally, physically active females are at a higher risk for menstrual dysfunction and LEA than sedentary females and further research is urgently needed to identify risk factors that may help detect women who need preventive intervention. Menstrual dysfunctions span a spectrum, making it imperative to identify subclinical menstrual disorders early, which could potentially prevent the progression to more severe menstrual dysfunction and thwart other medical conditions from occurring. However, current research is conflicting when evaluating menstrual dysfunction and LEA, potentially due to the lack of guidelines and high variation in protocols. Consensus needs to be achieved on the best practice for measuring energy availability, energy intake and EEE, as well as determining menstrual dysfunction. Additionally, further analyses of the association of menstrual cycle hormones and energy availability is needed to fully understand this relationship in active women. This proposal attempted to address many of these gaps in the literature.

CHAPTER III: RELATIONSHIPS OF SEX STEROID HORMONES AND ENERGY AVAILABLITY IN PHYSICALLY ACTIVE FEMALES

Abstract

Exercise alone does not appear to decrease estrogen and progesterone, but these hormones appear to be altered tangentially as a component of energy availability, with energy intake as a driving factor. Energy intake is reported to vary between 100-300 kcal across the menstrual cycle and is affected both directly and indirectly by estrogen and progesterone. In addition, fat-free mass (FFM) and resting metabolic rate (RMR) are the main drivers for energy intake and energy expenditure, as FFM is the body's most metabolically active tissue and RMR is a major component of energy expenditure (50-70%). OBJECTIVE: To examine the relationships of energy availability, estrogen, and progesterone in physically active females. METHODS: Healthy, exercising females (n=21; age 21 ± 3 years) not on oral contraceptives completed measures over two menstrual cycles. Daily saliva measurements were taken across both menstrual cycles to create hormonal profiles of estrogen and progesterone and determine if subclinical menstrual dysfunction was present (i.e., anovulation, luteal phase defect). Energy availability ([Energy intake (kcal) – Exercise Energy Expenditure (kcal)]/ FFM (kg)) was measured twice within one menstrual cycle, with energy intake recorded for seven days at two timepoints and exercise participation recorded with a heart rate monitor at the participant's discretion. The first timepoint (T1) started during menses between day (D) 2-4 and the second timepoint (T2) started between 5-8 days post ovulation. A laboratory visit occurred on the first day of each timepoint, where resting metabolic rate and body composition were measured. RESULTS: Most (71%, n = 15) of the physically active females were in a reduced energy availability state and 23% (n = 6) had subclinical menstrual dysfunction. Energy intake, energy availability, FFM, and RMR remained constant across the two timepoints despite that estrogen

and progesterone were significantly different (p = .003, p = .001). A repeated measures correlation revealed energy intake was correlated with the progesterone to estrogen ratio (P:E2) (p = .026, r= .321, 95% CI [0.04, 0.55]) in T1, but not progesterone or estrogen alone or in T2. When the components of energy availability and hormones were assessed, progesterone range was positively associated with FFM (T1 p = .015, r=.537; T2 p = .001, r = .674) and RMR (T2 p = .005, r = .605) yet T2 progesterone range, FFM, and RMR were all negatively associated with the average energy availability for the cycle (p = .032, r = -.479; p = .001, r = -.672; p = .009, r = -.558). CONCLUSIONS: These data suggest that physically active females are at risk for inadequate energy availability and subclinical menstrual dysfunction. Furthermore, this population did not follow typical patterns expected, yet when only eumenorrheic females were examined, the typical associations with hormones and FFM emerged. A higher progesterone to estrogen ratio was associated with higher energy intake during T1 but not progesterone or estrogen alone. Exercising females have lower hormone levels which may contribute to the discrepancies and warrants further investigation as much of the current research has focused on either highly active females or a sedentary population.

Introduction

It is widely accepted that exercising females (i.e., engaging in physical activity for health or fitness) have lower sex steroid hormones concentrations (i.e., estrogen, progesterone) when compared to sedentary individuals (Cumming et al., 1985; De Souza et al., 1998a; Ellison et al., 1987; Fisher et al., 1986; Jasienska et al., 2006; Lehmann et al., 1993; Matthews et al., 2012; Stoddard et al., 2006). However, exercise alone does not appear to decrease sex steroid hormones but alters hormones as a component of energy availability with energy intake as a driving factor (Williams et al., 2001). Energy availability is the amount of energy left after subtracting the energy cost of exercise relative to fat free mass (FFM) from energy intake. When

energy availability is inadequate (i.e., low energy availability (LEA)), disruptions to hormonal and metabolic systems occur that can lead to performance decrements, serious psychological (e.g., irritability, depression) and physiological health conditions (e.g., menstrual dysfunction, cardiovascular disease, decreased bone mineral density) (Mountjoy et al., 2014). While there is no accepted threshold of energy availability that induces negative health outcomes, when energy availability falls below 30 kcal/kg FFM/day, females have a 50% increased risk of menstrual dysfunction (e.g., decreased hormone concentrations, anovulation) (Lieberman et al., 2018). Nonetheless, it is unclear why menstrual function is disrupted by LEA in some females while menstrual function remains normal in others, indicating a need for more nuanced investigations of the components of energy availability (i.e., energy intake, FFM) to try to identify why estrogen and progesterone and ultimately menstrual function are altered.

Sex steroid hormones (i.e., estrogen, progesterone) are major determinants of premenopausal women's health status. Estrogen is the predominate female sex hormone and plays a role in almost every physiological system, including function of the reproductive, endocrine, urinary, nervous, immune, musculoskeletal and cardiovascular systems. Estrogen serves a protective role from multiple diseases in premenopausal women, therefore disruption of estrogen is associated with diseases such as cancer, cardiovascular disease, osteoporosis, neurodegenerative diseases, and metabolic disorders (Prossnitz & Barton, 2011). Progesterone regulates not only reproductive function but the nervous system and blood vessels as well. Further research is needed to fully understand the role of endogenous progesterone but research has demonstrated a neuroprotective effect in traumatic brain injury, stroke, and myelin repair (Franklin & ffrench-Constant, 2008; Sitruk-Ware & El-Etr, 2013; Stein & Wright, 2010). Additionally, the estrogen to progesterone ratio can influence core body temperature, sleep, and fluid volume (Giersch et al., 2021; Grant et al., 2020). Although it is theorized that energy

availability disrupts estrogen and progesterone through the GnRH pulse generator and luteinizing hormone (LH), the exact mechanism of how this occurs is unknown (Mountjoy et al., 2014) and the magnitude of the effect of LEA on estrogen and progesterone differs amongst individuals (Lieberman et al., 2018). Therefore, it is imperative to understand the relationship of estrogen and progesterone with energy availability, since decreases in estrogen and progesterone that result from LEA are likely to disrupt more than the menstrual cycle and reproductive system.

Energy intake can vary between 100-300 kcal across the menstrual cycle but there is no consensus regarding fluctuations in macronutrient intake across the cycle (Buffenstein et al., 1995; Chappell & Hackney, 1997; Hirschberg, 2012). Energy intake is the lowest in the follicular phase and peaks in the luteal phase (Barr et al., 1995; Buffenstein et al., 1995; Rocha-Rodrigues et al., 2021), potentially due to the energetic costs of reproduction (Jasienska, 2003) and sex hormones modulating energy intake (Asarian & Geary, 2006). Menstrual function may also influence energy intake as women with anovulatory cycles (i.e., decreased estrogen and progesterone) had unchanged energy intake across the menstrual cycle (Barr et al., 1995; Rock et al., 1996) further indicating that research should look at the associations between menstrual dysfunction, sex steroid hormones, and energy intake.

Estrogen and progesterone directly and indirectly affect energy intake. Estrogen is considered a potential appetite suppressant but the role of progesterone is not fully elucidated. Progesterone by itself does not change eating behavior in rats but stimulates appetite when estrogen is present (Hirschberg, 2012). Energy intake decreases when estrogen is high, with energy intake being lowest prior to ovulation (when estrogen is highest), and can result in an ~10% decrease in energy intake (Asarian & Geary, 2006). Additionally, postmenopausal women and women with ovariectomies demonstrated increased energy intake but estrogen

replacement therapy normalized energy intake (López & Tena-Sempere, 2015). Sex steroid conversions take place in muscle, indicating a need to evaluate FFM with estrogen and progesterone (Loucks & Horvath, 1985). Estrogen in skeletal muscles can stimulate growth of muscles and can inhibit inflammatory pathways. While decreased estrogen has been associated with decreased FFM, the effects of progesterone are less clear since the number of investigations specifically addressing progesterone are very limited (Y. J. Kim et al., 2016).

FFM and resting metabolic rate (RMR) are the main drivers for energy intake and energy expenditure (Blundell et al., 2020). FFM is the body's most metabolically active tissue (Mountjoy et al., 2014) that accounts for 60-70% of RMR. Furthermore, RMR is a major component of energy expenditure (50-70%), is strongly related to energy intake (Blundell et al., 2020), and can be an indicator of LEA, with values 20-40% less than expected in chronically energy-deficient women (Benton et al., 2020; Jasienska, 2003). The ratio of measured (i.e., indirect calorimetry) to predicted RMR (i.e., Cunningham, DXA) (mRMR:pRMR) is correlated with total triiodothyronine (TT_3) levels, which is a biomarker of energy deficiency. RMR ratio cutoff values below .90 and .94 for the Cunningham equation and DXA respectively, are indicators of LEA and have proven to be reliable measures regardless of menstrual function (i.e., amenorrhea, LPD) (Strock, Koltun, Mallinson, et al., 2020; Strock, Koltun, Southmayd, et al., 2020).

Exercise alone does not appear to disrupt estrogen and progesterone but these hormones appear to be altered tangentially via energy availability. Although previous research has investigated the relationship of LEA with lower estrogen and progesterone by proxy of menstrual dysfunction, direct associations with varying levels of energy availability with estrogen and progesterone across the cycle are less clear. Therefore, the purpose of the current study was to examine the relationships of energy availability, estrogen, and progesterone in physically active females. We hypothesized that: 1) lower energy availability, lower resting metabolic rate

ratio and lower levels of estrogen and progesterone, less range in hormone levels (less differences in min to max values) and more anovulatory cycles will be associated with lower fat free mass, regardless of fitness level (VO_{2peak}), and 2) energy intake will have a negative correlation with estrogen concentrations, with energy intake being high when estrogen concentrations are low at the beginning of the menstrual cycle.

Methods

Study Design

This longitudinal study took place across two menstrual cycles. Participants were required to attend seven in person visits (Figure 3.1 and Figure 3.2) at the Exercise Endocrinology Laboratory at the University of North Carolina at Greensboro (UNCG). Both menstrual cycles were used to collect data at home (e.g., menstrual cycle function, exercise) with in laboratory energy availability data (e.g., RMR) collected only in one menstrual cycle.

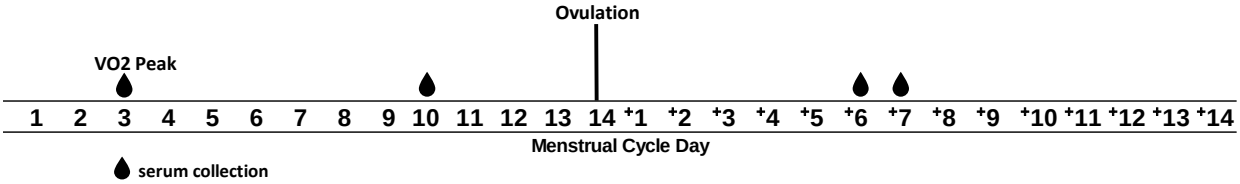
The baseline visit occurred prior to the participant's expected start date of their menstrual cycle. Participants were (1) consented, (2) received instructions on how to collect saliva and the vials for the first week, and (3) set up a Training Peaks (TP) account with instructions on how to record exercise. The TP account was also used as a study calendar (i.e., which days to record energy intake, ovulation testing), to track the menstrual cycle, and to annotate any major changes in a normal routine (i.e., illness, life or social stressor). If any major routine changes occurred, the participant was instructed to contact the researchers and a determination would be made to end, pause, or continue with the study. One participant was paused prior to her first visit due to illness and restarted on her next menstrual cycle while two participants ended the study after the first menstrual cycle due to time constraints.

The study assessed participants over two menstrual cycles, with different requirements per cycle and are listed below. Menstrual function was assessed for two consecutive menstrual

cycles. Saliva was collected every day, at home ovulation tests were done during the prescribed days (Table 3.1), and serum was obtained in both cycles. Participants did not perform the cycle requirements in the same order and were assigned based off equipment availability. The first visit had to be scheduled within 3 days of the start of the cycle and the shared equipment (i.e., metabolic carts) had limited availability therefore the first cycle was determined by the participants schedule and the equipment available. Nine participants started C1 first and 12 participants started C2 first.

Menstrual Cycle 1 (C1): Menstrual Function.

Figure 3.1. Research Study Design for Menstrual Cycle 1

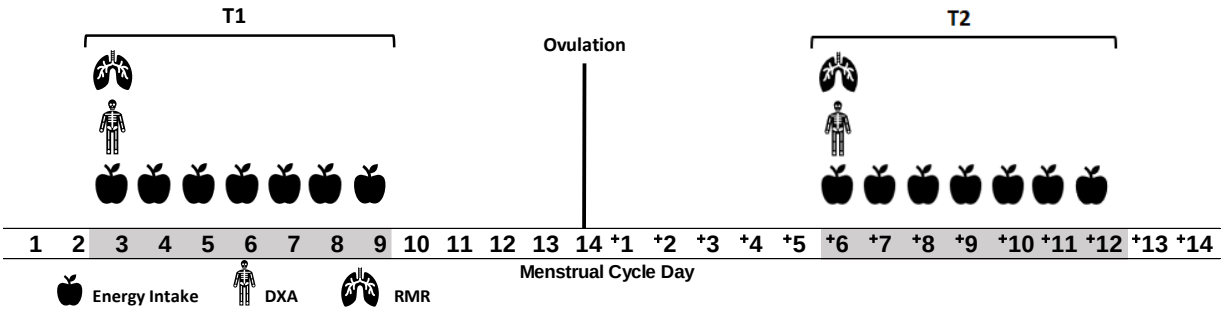


Note. This protocol is based off a 28-day cycle with ovulation occurring at D14. Collection days were adjusted per menstrual cycle and ovulation. VO_{2peak} and the first serum collection occurred between D2-D4 in the follicular phase; additional serum collections occurred between D9-11 and +D5-8. D1: day of onset of menses; +: post ovulation.

One menstrual cycle (C1) (Figure 3.1) consisted of four visits. The primary focus of this menstrual cycle was to assess menstrual function since it is recommended to assess menstrual function over a minimum of two cycles due to intra-variation across cycles (Elliott-Sale et al., 2021). The first visit occurred between day (D) 2-4 (i.e., early follicular) of a menstrual cycle that consisted of resting heart rate (RHR), blood pressure, serum collection, and VO_{2peak} testing. Additional visits to collect serum included another visit between D9-11 (i.e., late follicular) and two days between D5-D8 post ovulation (i.e., midluteal). Each visit took place between 0500-1000 and participants we instructed to fast for 8 hours prior to the visit. At home data collection consisted of daily saliva collection shortly after waking to assess hormones. At home ovulation tests were done during the prescribed days (Table 3.1).

Menstrual Cycle 2 (C2): Energy Availability.

Figure 3.2. Research Study Design for Menstrual Cycle 2

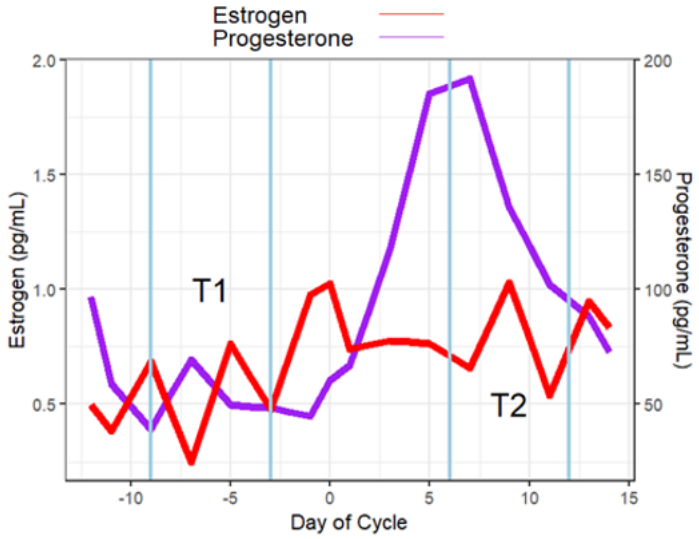


Note. This protocol is based off a 28-day cycle with ovulation occurring at D14. Collection days were adjusted per menstrual cycle and ovulation. Timepoint 1 (T1) started between D2-D4 in the follicular phase; Timepoint 2 (T2) started between +D5-8. D1: day of onset of menses; +: post ovulation.

The other menstrual cycle (C2) (Figure 3.2) consisted of two visits (i.e., timepoint 1 (T1) and timepoint 2 (T2)) and assessed energy availability and its components. Participants were required to fast for 12 hours prior to the visit and all visits occurred as close to waking as possible which took place between 0500-1000. Each visit marked the first day of the timepoint. T1 started in the follicular phase between D2-4 when estrogen and progesterone should be low, with estrogen starting to increase at the end of T1 while T2 started between D5-8 post ovulation when estrogen and progesterone should be high (Figure 3.3). Each visit (T1 and T2) consisted of RHR, blood pressure, resting metabolic rate (RMR) and dual energy x-ray absorptiometry (DXA) assessments. Additionally, serum was collected at the T2 visit. At home ovulation tests determined the start of T2 (i.e., D5-8 post ovulation). If ovulation did not occur based on our criterion time frame (Table 3.1), the cycle was considered anovulatory and T2 measurements occurred no later than two days after the final ovulation test. Energy intake was recorded for 7 days during each timepoint, starting the day of the laboratory visit unless an anovulatory cycle or late ovulation occurred, then energy intake recording started prior to the visit. Exercise was not prescribed and participants were encouraged to continue their normal exercise routine.

Therefore, exercise was conducted at the participant's discretion and recorded with an app and a heart rate monitor. Energy availability was calculated for each day energy intake was recorded in T1 and T2. At home data collection consisted of daily saliva collection shortly after waking to assess hormones.

Figure 3.3. An Example of the Salivary Estrogen and Progesterone Profiles Across a Single Menstrual Cycle for One Participant



Notes. Progesterone AUC: T1, 325.97; T2, 853.44. Progesterone mean: T1, 52.82; T2, 142.19. Estrogen AUC: T1, 3.19; T2, 4.56. Estrogen mean: T1, 0.54; T2, 0.76. Cycle days are centered on day of ovulation (day=0). Blue lines indicate start and end of each timepoint. T1, Timepoint 1, Day 4-10 (day -9 to -3 pre ovulation on graph); T2, Timepoint 2, Day 6-12 post ovulation. Values are presented as pg/mL.

Study Procedure

Participants. Participants were recruited from UNCG via word of mouth, emails, and flyers. This study was approved by the Institutional Review Board and all subjects provided written informed consent prior to enrollment in the study. Screening procedures consisted of a modified American College of Sports Medicine (ACSM) Exercise is Medicine Health History Questionnaire and questions regarding menstrual cycle, exercise, and medical health history. Inclusion criteria were as follows: (1) have a menstrual cycle between 21-50 days, (2) age 18-35 years, (3) a minimum of 2.5 hours of exercise per week (approximately 30-45 min per day or

more) and must be habitual exercisers for at least 6 months, (4) have no history of metabolic or cardiovascular diseases, eating disorders, or polycystic ovary syndrome (PCOS), (5) not take any hormonal contraceptives for 3 months prior to the start of the study or take any medications that would alter the metabolic or reproductive hormones (e.g. anxiety, depression, stimulants), (6) must not currently be using tobacco products (e.g. smoking, vaping), (7) not be actively dieting to lose weight, (8) are not or do not plan to become pregnant during the duration of the study and (9) have no internal metal (e.g. hip replacement, fixation of the spine).

Menstrual Cycle Characteristics

A menstrual cycle was defined as the first day of menses (Day 1) until the day prior to onset of the next menses. For this study, the follicular phase was defined as D1 through the day of the LH surge (i.e., indicating ovulation) regardless of length and the luteal phase is the day after the LH surge until the day before the onset of menses regardless of length. Luteal phase days are referred to as days post ovulation. If ovulation did not occur, phases could not be determined.

Participants verified having consistent menstrual cycles every 21-50 days or were asked to track one menstrual cycle prior to starting the study to determine eligibility. The menstrual cycle length 21-50 days was selected because even though a ‘normal’ menstrual cycle is between 21-35 days, it is also considered normal for menses to fluctuate up to a cycle length of 45 days for the first few years after menarche (Klein et al., 2019). Additionally, this allowed participants with potentially subclinical menstrual dysfunction (e.g., anovulation) to participate. Menses was logged into Training Peaks for both menstrual cycles and menstrual cycle function and history was assessed once with the Low Energy Availability in Females Questionnaire (LEAF-Q) (Melin et al., 2014) prior to the first visit. Participants reported age of menarche (i.e., first menses) and gynecological age was determined from subtracting current age from age of

menarche. Participants were instructed to contact the research team on D1 of each menstrual cycle in order to schedule subsequent visits.

Menstrual Cycle Classification

Ovulation was measured in both menstrual cycles and testing days were adjusted based on the length of the previous menstrual cycle (Table 3.1). Ovulation testing days were selected as the highest probability of ovulation occurring within that menstrual cycle length (Soumpasis et al., 2020). Participants sent a text message via WhatsApp to the research team daily that included a picture of that day's ovulation test and continued testing daily until the research team confirmed a positive ovulation or the number of testing days has been reached (see Table 3.1). If the participant failed to text a picture for two days, the research team contacted the participant. At home ovulation testing is proven to be accurate but ovulation was also confirmed by serum progesterone values post conclusion of the study. Failure to receive a positive ovulation test and failure to obtain either a serum progesterone > 3.0ng/mL (American Society for Reproductive Medicine and the Society for Reproductive Endocrinology and Infertility, 2021) or an increase of salivary progesterone that was 2 SD above the follicular mean post ovulation (Ellison, 1988), resulted in the cycle being classified as anovulatory. A luteal phase deficit (LPD) was defined by < 10 days post ovulation to the start of the next menses (De Souza et al., 1998b). Eumenorrheic cycles are defined as ovulatory cycles with luteal phase length greater than 9 days. Menstrual dysfunction refers to anovulatory and/or LPD cycles.

Table 3.1. Ovulation Testing Days based on MC length

MC Length	Testing Days
21-23	5-18
23-28	7-20
29-35	8-25
36-50	8-30

Anthropometrics

Nude body mass was measured to the nearest 0.1 kg on a digital scale (WB-800S Plus; Tanita Corporation, Tokyo, Japan) at each visit while height was measured by a wall mounted stadiometer (Model216; Seca, Chio, CA) to the nearest 0.5 cm at the beginning of the study. Body mass was also calculated via the DXA analyses. Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2).

Body Composition (DXA)

Dual-energy X-ray absorptiometry (DXA) (Lunar Prodigy Advance, GE Healthcare, Madison, Wisconsin) was utilized to determine whole-body composition by a certified operator. Participants were fasted for a minimum of 2 hours prior and a pregnancy test was administered prior to the scan to confirm that the participant was not pregnant. Each participant wore a gown and removed all metal before the scan, which was performed twice in one menstrual cycle (C2) (i.e., T1, T2) to determine if body composition changed across the menstrual cycle. The DXA scanner has a <1% coefficient of variation for body composition measurements. Body composition was calculated using enCORE 2011 software (version 13.60) by one researcher.

Energy Availability

Energy availability describes the amount of energy available for metabolic functions after exercise energy expenditure (EEE) is subtracted from energy intake, and is expressed relative to fat-free mass (FFM) (Loucks et al., 1998).

Energy availability = [Energy intake (kcal) – EEE (kcal)]/ FFM (kg)

Although there is no widely accepted threshold for energy availability, the recommendation for adequate energy availability is ≥ 45 kcal/kg FFM/day, whereas ≤ 30 kcal/kg FFM/day has been recommended as the threshold for low energy availability.

Energy availability was calculated for 7 consecutive days during T1 and T2 during C2. The average FFM from T1 and T2 from the DXA analysis was used. Energy intake and exercise energy expenditure collection procedures are listed below. Daily energy availability was used in the repeated measures correlation while the 7-day average was used in the Pearson correlation.

Low Energy Availability in Females Questionnaire (LEAF-Q)

The Low Energy Availability in Females Questionnaire (LEAF-Q) was administered once via Qualtrics prior to the first visit. The LEAF-Q is a validated screening tool comprised of 25 questions that has a 78% sensitivity and 90% specificity. The LEAF-Q assesses risk for low energy availability (LEA) though injuries, gastrointestinal (GI) and menstrual function. Participants with a total score of ≥8 are considered at risk for LEA (Melin et al., 2014) (Appendix B).

RMR and RMR Ratio

RMR was assessed with indirect calorimetry (TrueOne 2400; Parvo Medics Inc., Salt Lake City, UT) during T1 and T2 in C2. Participants were instructed not to perform exercise or intake alcohol 24 hours prior to testing and to fast overnight (≥12 hours). RMR was measured by a ventilated hood that was placed over the head for 30 minutes, after a 20–30-minute rest period. The participant was instructed to lay as still as possible and not to sleep. The first five minutes of data were discarded to allow for stabilization and a total of 20 minutes was used to calculate RMR.

Predicted RMR was measured with data from the DXA analysis (i.e., organ tissue mass of the brain, skeletal muscle, bone, adipose tissue, residual mass) with methods outlined by Hayes et al. (M. Hayes et al., 2002). A calculation of measured RMR to predicted RMR

(mRMR:pRMR) assessed energy availability, with a cutoff value of ≤ 0.94 for the DXA equation indicating low energy availability (Strock, Koltun, Southmayd, et al., 2020).

Energy Intake

Energy intake was assessed for seven days at two timepoints (i.e., T1, T2) across one menstrual cycle (C2). Due to subject burden and to increase compliance, only two timepoints were assessed and seven days was chosen to best evaluate the association of energy intake and exercise. Energy intake was self-logged in MyFitnessPal and began the day of each follicular and luteal phase laboratory visit, whenever possible. If the participant was anovulatory or had a late ovulation, energy intake was recorded prior to the T2 visit to allow the full seven days to be logged. Participants received detailed instructions from the researcher on how to log all food and beverages as well as a handout with portion sizes. The same researcher also confirmed MyFitnessPal entries at the next visit to ensure accuracy. MyFitnessPal is a validated smartphone app with reliable dietary analysis (Evenepoel, Clevers, Deroover, Loo, et al., 2020; Evenepoel, Clevers, Deroover, Matthys, et al., 2020; Teixeira et al., 2018). Total energy intake and macronutrient composition were calculated from data from MyFitnessPal. Total daily energy intake was used for the repeated measures correlation and the 7-day total energy intake and macronutrients average was used for the Pearson correlation.

Exercise

Participants were given a Polar H10 heart rate monitor (Polar H10 Heart Rate Sensor; Polar Electro Inc., Bethpage, NY) to wear during all purposeful exercise greater than 10 minutes in duration during C2. Purposeful exercise can include activities such as jogging, strength training, etc. but not daily living activities such as house cleaning. The heart rate monitor was linked to a recording device of the participants choice (e.g., Polar Beat, Garmin, Apple) that automatically uploaded data to the Training Peaks (TP) app. Ratings of perceived exertion

(RPE) was recorded using a built-in function of TP that utilized a modified sliding scale of 0-10 for each exercise session.

Calculation of exercise energy expenditure used training heart rate divided into 7 heart rate zones equally distributed between resting heart rate (RHR) and maximum heart rate. For each heart rate zone, the metabolic equivalent (MET) was calculated using the heart rate index method ($6*(HR_{absolute}/HR_{rhr})-5$). This method has been validated and shown to be an accurate measure of VO_2 at different intensities (KANG et al., 2020; Wicks et al., 2011; Wicks & Oldridge, 2016). A MET is approximately equal to a resting value of 3.5 ml/kg/min but this varies by participant and females typically have lower values (Byrne et al., 2005). Therefore, corrected METs were calculated using the measured resting VO_2 (MET * 3.5ml/kg/min / resting VO_2 ml/kg/min). Kilocalories from exercise were quantified using the corrected METs multiplied by exercise duration (min) and weight (kg). MET values contain resting values, therefore measured resting energy expenditure (kilocalories/min) were subtracted from the exercise kilocalories to obtain the correct exercise energy expenditure (Reed et al., 2015).

If heart rate was not recorded, the participant logged a description of the activity, duration, intensity, and RPE in training peaks which was used to determine the appropriate METs with the compendiums of physical activities (Ainsworth et al., 2011). Corrected METs was calculated prior to calculating kilocalories as described above by the same researcher.

Physical Activity

Wrist-worn actigraphy (ActiGraph GT9X Link) assessed total daily activity for 7 days, starting the day of the T1 visit. Participants were instructed to continuously wear the accelerometer on their nondominant wrist, except for activities that involved water (i.e., swimming, showering). Accelerometers were programmed and downloaded using Actigraph software. The raw data analyses were performed with R- package GGIR (Hees et al., 2013) that

was expressed in gravitational equivalent units called milli-gravity (*mg*, where 1000*mg* = 1*g* = 9.81 m/s^2). There is no criterion for categorizing total physical activity (i.e., low, high) when calculating the physical activity with raw data. Therefore the data is used to assess total physical activity, with higher values indicating higher physical activity. To be included in the analyses, participants needed four days that included a minimum of 16 hours of wear time. Daily physical activity was used in the repeated measures correlation, while the 7-day average of daily physical activity was used for the Pearson correlation.

Maximal Aerobic Exercise Testing (VO_{2peak})

Maximal aerobic exercise testing (VO_{2peak}) was administered between D2-4 in C1. Participants were tested in a climate control chamber (CES-5-43; CANTROL International Inc., Markham, Ontario) set at 20℃ and 40% humidity. Oxygen uptake and related gas exchange was captured using indirect calorimetry (TrueOne 2400; Parvo Medics Inc., Salt Lake City, UT) and included VO_2 and respiratory exchange ratio (RER). A heart rate chest strap (Polar H10 Heart Rate Sensor; Polar Electro Inc., Bethpage, NY) measured heart rate every minute. A 5-minute warm up at a self-selected pace was allotted on a motorized treadmill (T150; h/p/cosmos, Munich, Germany). The maximal exercise test was performed at a self-selected pace where the grade of the treadmill increased 1% every minute of the test until the participant reached volitional fatigue. During the test, the rating of perceived exertion (RPE) was collected every other minute via the Borg 6-20 scale (Borg, 1998).

Hormones

Blood Collection and Preparation. Participants reported to the UNCG Exercise Endocrinology Laboratory between the hours of 0500-1000 for one visit during the following ranges: D2-4, D9-11, and two consecutive days between D5-8 post ovulation in one menstrual cycle then again between D5-8 post ovulation (i.e., T2) in the other menstrual cycle for a total of

5 blood samples. Participants were instructed to be fasted for 8 hours prior to arrival. Approximately 10mL of blood was collected in a serum blood collection tube. Blood samples were allowed to clot for 20 min at room temperature then centrifuged at 3000 rpm for 15 min at 4°C. The serum samples were aliquoted into multiple 2 mL polyethylene storage tubes and frozen at -80°C.

Saliva Collection and Preparation. Saliva collection was chosen to measure the daily hormonal profile for two menstrual cycles to reduce invasiveness and increase participant compliance. Measurements of E2 and progesterone in saliva have a strong correlation with serum concentrations in premenopausal women and additionally are stable at room temperature for a few days and at -20°C long-term (Bellem et al., 2011). Serum was collected to confirm the correlation between saliva and serum.

Saliva was collected every day via passive drool using polyethylene storage tubes with straws supplied to the participant by the investigator. Participants were instructed to collect their saliva immediately after waking and to refrain from brushing teeth, eating, or drinking until the sample is collected. Participants stored saliva samples in a home freezer (-20°C) until they were returned to research team at every visit, then the samples were stored at -80°C. Saliva samples were thawed at room temperature and then centrifuged at 13,000 rpm for 15 minutes prior to assay. Salivary collection occurred every day but to minimize cost, only every other day was analyzed.

Serum and Saliva Measurements. Estrogen (17-β estradiol) and progesterone were quantified in serum and saliva samples by enzyme immunoassay (Immuno-Biological Laboratories, Minneapolis, MN (serum); Salimetrics, Carlsbad, CA (saliva)). All hormone determinations were assayed in duplicate with all samples from a given participant on the same assay whenever possible. The sensitivity of the serum and saliva E2 assays are < 1.399 pg/mL

and 0.1pg/mL and the progesterone assays have a sensitivity of 0.045 ng/mL and 5pg/mL, respectively. Samples were reanalyzed if a coefficient of variation was > 25% for saliva and > 20% for serum. The intra-assay coefficients of variation for low and high controls were 9.6% and 3.3% (saliva progesterone), 3.1% and 5.9% (saliva estrogen), and 19.2% and 5.7% (serum estrogen) respectively. Inter-assay coefficients of variation for low and high controls were 19.6% and 34.2% (saliva progesterone), 13.5% and 16.6% (saliva estrogen), and 22.9% and 22.5% (serum estrogen) respectively. Serum and salivary hormones were used to verify ovulation and for salivary hormonal profiles for each menstrual cycle.

Statistical Analysis

Statistical analyses were conducted using SPSS Statistics 24 (IBM, Armonk, NY, USA) and R Statistical Software (v2022.12.0; R Core Team 2021). Data was summarized as mean ± SD.

Estrogen and progesterone were analyzed separately and as progesterone:estrogen (P:E2) ratio. Salivary hormonal profiles were created by quantifying each hormone every other day across two menstrual cycles. Area under the curve (AUC) was calculated using the trapezoidal method for the entire cycle, T1, and T2 to determine the total value of the free concentration of each hormone. Total AUC for each menstrual cycle was calculated off the length of each cycle and was not normalized. Hormone values were imputed for T1 and T2 to ensure the AUC equaled 7 days for every participant at each timepoint. Additionally, the mean, minimum and maximum, difference between minimum and max (i.e., hormone range), and difference from the mean (i.e., hormone variability) values of each hormone were assessed per menstrual cycle. The hormone range was the difference between the maximum and minimum value of each cycle. Variability was the individual difference from the average mean of all participants per cycle. The estrogen peak was defined by the highest concentration in the

follicular phase and the peak mean was calculated by averaging 2 days prior and 2 days after the peak value. The progesterone peak was the highest concentration in the luteal phase, and the peak mean was calculated by averaging 2 days prior and 2 days after the peak value. If anovulatory, follicular was considered the first half of the cycle and the luteal phase was the second half to determine the peaks only. Otherwise, no ‘phases’ were assigned for anovulatory cycles.

Estrogen and progesterone were analyzed separately per menstrual cycle (i.e., variability, AUC) then a paired t-test was conducted to determine if there was a difference between cycles. A paired t-test was performed to test if resting metabolic rate, body composition, and energy availability changed within one menstrual cycle. If no difference was detected with the paired t-test, the average was taken from the two measurements and used for analyses when appropriate.

A repeated measures correlation determines within-individual association for measures occurring on multiple occasions for multiple individuals. A repeated measures correlation determined the relationship between daily energy intake, physical activity, and energy availability for seven days at T1, then again at T2. A repeated measures correlation also assessed the relationship of estrogen, progesterone, and P:E2 with energy intake every other day in each timepoint. Hormones were measured every other day and the corresponding energy intake from that day was used and was assessed in both T1 and T2.

A Pearson correlation coefficient determined the relationship of fat free mass with energy availability, estrogen, progesterone, hormone variability, hormone range. The Pearson correlation identified significant correlations with FFM and the significant variables were used in a multiple step-wise linear regression analyses to determine which measures predicted fat free

mass, while controlling for VO_{2peak}. An independent t-test assessed if FFM was different between eumenorrheic females and females with subclinical menstrual dysfunction.

Results

Demographic and Reproductive Characteristics

The demographic and reproductive characteristics are shown in Table 3.2 and Table 3.3. Twenty-one participants completed the study, with 18 participants completing both menstrual cycles consecutively. Three participants only completed one cycle (C2) due to time constraints. Two additional participants started the study (C1) but stopped the study prior to finishing a full menstrual cycle because of time constraints and an irregular menstrual cycle. Overall, 21 participants completed the study but due to one participant having low compliance with collecting saliva, only 20 participants are used for analyses. Participants identified themselves as Caucasian (43%), Hispanic (19%), African-American (33%) and an unlisted ethnicity (5%). All participants were nulliparous and underwent menarche prior to 15 years of age. Strength training was the primary mode of physical activity for most of the participants (81%). Many participants engaged in multiple activities, including running (57%), yoga (29%), indoor cycling (31%), soccer (19%), Zumba (14%), and dance (10%).

Table 3.2. Demographic and Reproductive Characteristics of Participants

	n=21
Age (years)	21 ± 3
Height (cm)	163.2 ± 4.5
BMI (kg/m^2)	23.8 ± 4.0
Age of menarche (years)	11 ± 1
Gynecological age (years)	9 ± 3
$VO2_{peak}$ (ml/kg/min)*	39.9 ± 1.4
Physical activity (*mg*/d)	40.8 ± 14.7
LEAF-Q	5.2 ± 3.8

Values are mean ± SD. *****n=19; *mg/d*, milli-gravity per day; LEAF-Q, Low Energy Availability in Females Questionnaire. A LEAF-Q score of ≥ 8 indicates at risk for low energy availability.

Table 3.3 summarizes the demographics measured in T1 and T2. Weight, BMI, body fat %, and fat free mass did not change across the cycle ($p > .05$). The mean of both timepoints is listed and was used in analysis since there were no difference between timepoints.

Table 3.3. Demographic Characteristics at T1 and T2

	T1	T2	p	Mean
Body Mass (kg)	63.2 ± 11.4	63.2 ± 11.5	.756	63.22 ± 11.4
Body fat (%)	30.1 ± 8.7	29.9 ± 9.1	.509	30.0 ± 8.9
Fat free mass (kg)	43.4 ± 5.8	43.5 ± 5.8	.671	43.4 ± 5.8

Values are mean ± SD. All value are n=21.

Energy Availability Characteristics

Energy availability characteristics are summarized in Table 3.4. Energy availability measures were obtained during a single menstrual cycle only, at two timepoints: timepoint 1 (T1) that started between D2-4 after the start of the menstrual cycle, when estrogen and progesterone are low (typically, the early follicular phase) and timepoint 2 (T2) that started between D5-8 post ovulation when estrogen and progesterone are high (typically, the luteal phase). Despite the use of home ovulation tests, retrospective serum hormone analysis could not confirm all participants met ovulation and/or luteal criterion measures. Although T1 is definitively representative of the follicular phase, T2 did not always definitively represent the luteal phase and thus, T1 and T2 will be used instead of follicular phase and luteal phase. No measures of energy availability changed across the cycle ($p > .05$). The mean of both timepoints is listed and was used in analysis since there were no difference between timepoints. Most participants (71%; 15/21) were in a reduced energy availability state, with 19% (4/21) classified as low energy availability (< 30 kcal/kg FFM) while only 10% (2/21) were above the recommended 45 kcal/kg FFM. Energy availability, energy intake, macronutrients, RMR, and RMR ratio did not change from T1 to T2 ($p >.05$). No participants were deemed to have LEA (<.94) by the RMR ratio. Yet, the LEAF-Q classified six participants at risk (total score ≥8) for LEA. Only one participant was classified as LEA by both the < 30kcal/kg FFM value and the

LEAF-Q. An a priori analysis with an independent t-test looked at the characteristics of five participants with the highest energy availability and five with the lowest energy availability. Females with the lowest energy availability had significantly larger values for weight (kg) (74.1 ± 14.8 vs 55.6, p = .047), FFM (kg) (48.6 ± 6.9 vs 38.7, p = .031), BMI (27.3 ± 20.8 vs 20.8 ± 2.0, p = .032) and less total energy intake (kcal) (1443.6 ± 152.1 vs 1886.1 ± 247.3, p = .012) compared to the females with the highest energy availability. No difference was found in body fat percentage (33.3 ± 9.3 vs 28.1 ± 11.3) or total duration of exercise hours per week (3.7 ± 2.2 vs 2.7 ± .8).

Table 3.4. Energy Availability Characteristics at T1 and T2 Across One Menstrual Cycle

	T1	T2	p	Mean
EA (kcal/kg FFM/day)	35.8 ± 11.5	35.8 ± 7.5	.993	35.8 ± 8.8
Energy Intake (kcal/d)	1682.6 ± 390.3	1667.6 ± 179.8	.855	1675.1 ± 240.1
Carbohydrate (% kcal/d)	45.0 ± 6.2	44.8 ± 7.2	.849	44.9 ± 6.2
Fat (% kcal/d)	38.5 ± 6.6	36.3 ± 7.5	.298	37.4 ± 5.4
Protein (% kcal/d)	17.4 ± 4.3	16.7 ± 5.7	.430	17.0 ± 4.7
Protein (g/kg/d)	1.2 ± 0.5	1.1 ± .37	.860	1.2 ± 0.4
Exercise duration (hour/week)	3.2 ± 1.8	2.5 ± 1.9	.190	2.9 ± 1.4
EEE (kcal/d)	168.9 ± 142.1	143 ± 152.0	.255	156.0 ± 138.3
RMR (kcal/d)	1406.7 ± 141.2	1400.3 ± 183.1	.798	1403.5 ± 153.4
DXA pRMR (kcal/d)	1241.8 ± 134.4	1248.3 ± 128.3	.515	1245.0 ± 129.4
mRMR : pRMR	1.1 ± 0.1	1.1 ± 0.1	.638	1.1 ± 0.1

Values are mean ± SD. d, day; EA, energy availability; EEE, exercise energy expenditure; RMR, resting metabolic rate; mRMR, Measured RMR; pRMR, predicted RMR. All values are n=21.

Menstrual Cycle Characteristics

Menstrual cycle characteristics are displayed in Table 3.5 for the participants that completed both menstrual cycles (n=18). When evaluating both menstrual cycles together (n=21 first menstrual cycle, n=18 second menstrual cycle), 39 menstrual cycles were assessed that ranged from 21-38 days. 77% (30/39; n=15) of cycles were categorized as ovulatory and 23% of women (n=6) had subclinical menstrual disturbances and had cycles that were either anovulatory (6/39) or LPD (3/39). Three participants experienced menstrual dysfunction for both

cycles. One participant was anovulatory for both cycles and the other two had one anovulatory and one LPD cycle. The remaining three participants only had menstrual dysfunction for one of the two cycles. Out of the six females that experienced menstrual dysfunction, only three were identified at risk for low energy availability. Only one female with menstrual dysfunction was classified at risk for low energy availability with the LEAF-Q and < 30 kcal/kg FFM/d threshold. One additional female had energy availability < 30 kcal/kg FFM/d and the third female was classified at risk for low energy availability by the LEAF-Q. Neither method identified three females with menstrual dysfunction at risk for low energy availability.

Total menstrual cycle days, days in the follicular and days in the luteal phase, and day of ovulation did not change between the two menstrual cycles ($p > .05$).

Table 3.5. Menstrual Cycle Characteristics in Two Menstrual Cycles

	Cycle 1	Cycle 2	p	Mean
Menstrual cycle length (d)	30.0 ± 3.9	28.6 ± 3.0	.381	29.6 ± 3.4
Day of ovulation (d)	15.3 ± 3.7	16.4 ± 3.9	.941	15.8 ± 3.8
Anovulatory cycles	n=3	n=3		
LPD cycles	n=0	n=3		

Values are mean ± SD. Menstrual cycle characteristics are listed in the order the cycles occurred. Only participants that completed both menstrual cycles are presented (n=18), except for day of ovulation n=12. LPD, luteal phase defect; AUC, area under the curve.

Hormone Characteristics

Salivary Hormone Characteristics. Salivary hormone characteristics are shown in Table 3.6 and Table 3.7. Note that hormone values in Table 3.6 provide the values for the entire cycle (C1 and C2; not standardized for cycle length), while Table 3.7 presents the mean values at T1 and T2 for the seven days included in these assessment times (standardized AUC for 7-days).

Displayed in Table 3.6 for both menstrual cycles are the estrogen and progesterone area under the curve (AUC), variability, range, and peak mean. These values were similar in both menstrual cycles ($p > .05$). Progesterone and estrogen total AUC for the first menstrual cycle

are shown in Figure 3.4 and 3.5, respectively. Three participants salivary hormonal profiles for two consecutive menstrual cycles are displayed in Figure 3.6.

Table 3.6. Salivary Hormone Characteristics in Two Menstrual Cycles

	Cycle 1	Cycle 2	p	Mean
Salivary Progesterone*				
Total AUC	6092.7 ± 3717.3	6165 ± 929.2	.877	6128.8 ± 3653.9
5-d peak	331 ± 179.0	345.3 ± 189.4	.670	338.6 ± 172.8
Total mean	231.74 ± 139.6	228.4 ± 142.9	.858	230.1 ± 136.1
Variability	-12.3 ± 139.6	.0004 ± 142.9	.514	-6.1 ± 136.1
Minimum	76.43 ± 84.0	68.8 ± 86.6	.672	72.6 ± 77.2
Maximum	440.5 ± 226.1	480.7 ± 246.6	.265	460.6 ± 225.5
Range	364.1 ± 190.7	411.9 ± 204.2	.081	388.0 ± 190.3
Salivary Estrogen*				
Total AUC	41.7 ± 13.2	44.14 ± 15.8	.413	42.9 ± 13.2
5-d peak	1.8 ± .5	1.8 ± .6	.556	1.8 ± 0.5
Total mean	1.6 ± .4	1.6 ± .5	.577	1.7 ± .5
Variability	-.04 ±.4	-.003 ± .6	.659	.002 ± 74.8
Minimum	.92 ± .5	.76 ± .5	.144	.84 ± .4
Maximum	2.5 ± 0.6	2.4 ± .8	.909	2.4 ± 0.6
Range	1.6 ± 0.5	1.7 ± .7	.672	1.6 ± .5

Values are mean ± SD. Menstrual cycles are listed in the order the cycles occurred. Only hormone values for participants that finished two cycles with adequate hormone collection are presented, n=17. * all saliva concentrations are expressed as pg/mL. All hormones are for the entire menstrual cycle except for peak. AUC is presented as total, not normalized for days. Progesterone peak, average of 5 days centered on the peak post ovulation. Estrogen peak, average of 5 days centered on peak pre-ovulation. AUC, area under the curve.

Table 3.7 summarizes hormones for T1 and T2. Estrogen (8.27 ± 4.4 vs. 10.55 ± 4.2, p = .003) and progesterone (1197.4 ± 932.0 vs. 1765 ± 1033.9, p = .001) were lower in T1 compared to T2.

Table 3.7. Salivary Hormone Characteristics in T1 and T2 Across One Menstrual Cycle. Hormone values at T1 and T2 represent 7-day standardized values for progesterone and estrogen.

	T1	T2	p
Progesterone AUC	1197.4 ± 932.0	1765.17 ± 1033.9	**.001**
Progesterone range	154.3 ± 109.4	226.8 ± 136.4	**.027**
Progesterone minimum	128.4 ± 122.8	180.9 ± 137.1	.094
Progesterone maximum	282.7 ± 174.1	407.7 ± 223.0	**.001**
Estrogen AUC	8.3 ± 4.4	10.5 ± 4.2	**.003**
Estrogen range	.8 ± .6	1.0 ± .7	.176
Estrogen minimum	1.0 ± .6	1.2 ± .5	**.017**
Estrogen maximum	1.8 ± .8	2.2 ± .9	**.004**

Values are mean ± SD. d, day; AUC is presented as total for each timepoint, not normalized for days.
*all hormones are presented as pg/mL, n=20. T1, timepoint 1; T2, timepoint 2; AUC, area under the curve.

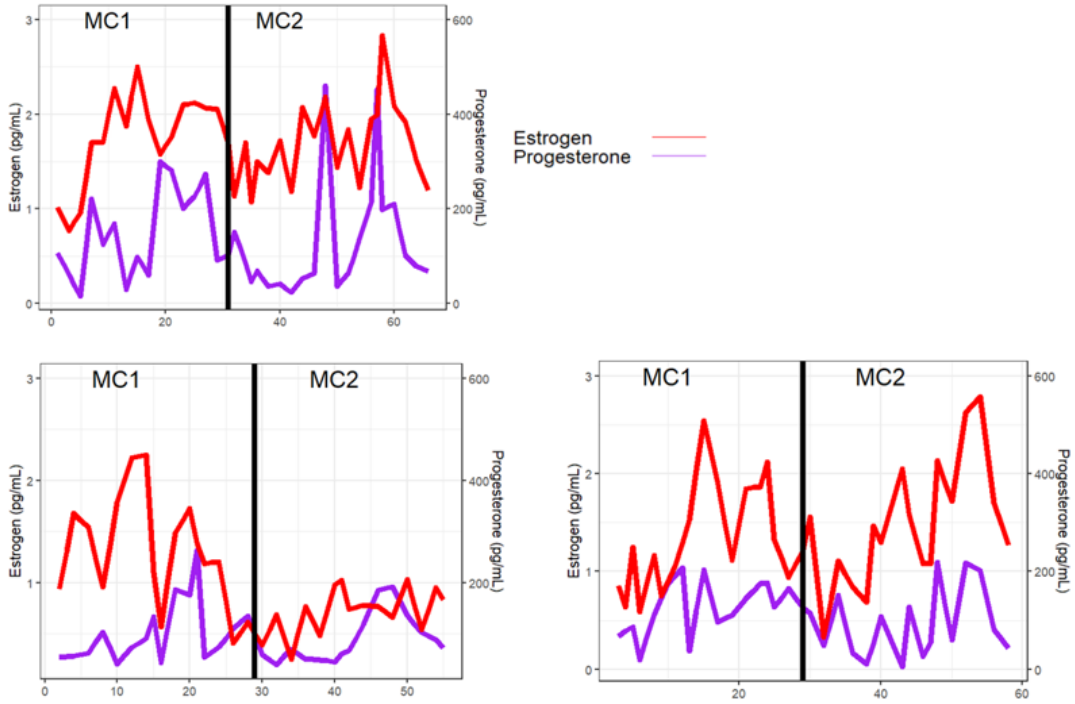
Figure 3.4. Variation of Progesterone (pg/mL) Total Area Under the Curve (AUC) in the First Menstrual Cycle by Participant

Note. X-axis represents progesterone AUC (pg/mL). Y-axis represents day of menstrual cycle and hormones are centered on the day of ovulation (day=0) unless the cycle was anovulatory (Participant 20, 21, 23). Total menstrual cycle days for each participant are presented and vary per participant.

Figure 3.5. Variation of Estrogen (pg/mL) Total Area Under the Curve (AUC) in the First Menstrual Cycle by Participant

Note. X-axis represents estrogen AUC (pg/mL). Y-axis represents day of menstrual cycle and hormones are centered on the day of ovulation (day=0) unless the cycle was anovulatory (Participant 20, 21, 23). Total menstrual cycle days for each participant are presented and vary per participant.

Figure 3.6. Estrogen and Progesterone is shown for Three Participants Across Two Eumenorrheic Menstrual Cycles to Display the Large Intra- and Inter-individual Variation in Menstrual Cycles.



Note. Days are labeled as they occurred and are not normalized, with the black line separating menstrual cycle 1 (MC1) from menstrual cycle 2 (MC2).

Serum and Saliva Hormone Characteristics. Serum hormone characteristics are displayed in Table 3.8, while the serum with matching same day saliva correlations for progesterone and estrogen are displayed in Table 3.9, 3.10, and 3.11. Serum and saliva characteristics are shown to illustrate the complexity of menstrual cycle hormone profiles and to show the values that determined menstrual function. Furthermore, little research has been performed across the menstrual cycle with salivary hormone concentrations so the comparison of serum (i.e., gold standard) to salivary hormones is shown. Four serum samples were collected in C1 and one serum sample was collected in C2. Serum samples were used to confirm menstrual function and since only one serum sample was collected in C2, serum hormones are not used for analysis for the primary aims. Not all five-serum samples were successfully collected in each participant, with only 14 participants with all five serum samples.

In addition, even though saliva was collected every day, participants missed days or had low volume. All participants with serum values are listed in Table 3.8 and serum and saliva comparisons in Table 3.9, regardless of the total days collected. Participants with five matching serum and saliva samples (n=6) are listed in Table 3.10 and Table 3.11 are the serum saliva correlations for participants that had less than 5 corresponding serum and saliva samples. Four out of six progesterone serum and saliva correlations were in the expected range of .75 to .93. Only two correlations were significant, but this could be due to only having five participants with five matching samples. The serum and saliva estrogen correlations were much lower than the expected .76 to .89 range, with no significant correlations. The correlation of all saliva and serum samples for progesterone ($r = .276$, $p = .022$) was significant but not for estrogen ($r = .05$, $p = .67$).

Table 3.8. Serum Characteristics

	T2 (C2)	D2-4 (C1)	D9-11 (C1)	D5-8+ (C1_1)	D5-8+ (C1_2)
Serum Progesterone (ng/mL)	7.7 ± 7.9	.88 ± .32	.90 ± .4	6.2 ± 3.8	7.7 ± 7.5
Serum Estrogen (pg/mL)	132.6 ± 59.5	89.7 ± 66.7	143.0 ± 64.5	130.1 ± 86.7	145.7 ± 64.1

Values are mean ± SD. +, post ovulation. T2, n=16; D2-4, n=18; D9-11, n=14; D5-8+(1), n=17; D5-8+(2), n=16. T2 was taken during one menstrual cycle (C2) between D5-8+ and the remaining timepoints were taken in the other menstrual cycle (C1). D5-8+(C1_1) and (C1_2) were taken on consecutive days whenever possible.

Table 3.9. Serum Characteristics with Same Day Saliva Values

	.	D2-4 (C1)	D9-11 (C1)	D5-8+ (C1_1)	D5-8+ (C1_2)
Serum Progesterone (ng/mL)	7.5 ± 8.1	.87 ± .4	.89 ± .4	5.8 ± 3.5	7.2 ± 6.7
Saliva Progesterone (pg/ml)	346.7 ± 238.5	217.5 ± 162.5	179.8 ± 177.1	286.5 ± 247.9	432.8 ± 289.4
% free / total	4.6	25.0	20.1	4.9	6.0
Serum Estrogen (pg/mL)	140.8 ± 58.8	92.9 ± 69.2	143.0 ± 64.5	129.7 ± 86.5	145.8 ± 70.8
Saliva Estrogen (pg/mL)	1.77 ± .6	1.4 ± .6	1.6 ± .6	1.9 ± .8	1.88 ± 1.0
% free / total	1.3	1.5	1.1	1.5	1.3

*p < .05. +, post ovulation Values presented are based of serum and saliva samples obtained on the same day. Only participants with the maximum 5 corresponding serum and saliva samples are presented in this table. Even though saliva was collected daily, participants missed days or gave low sample volumes. Additionally, not all participants had all 5 serum samples. Progesterone: T2, n=14; D2-4, n=15; D9-11, n=13; D5-8+(1), n=16; D5-8+(2), n=11; Estrogen: T2, n=14; D2-4, n=16; D9-11, n=14; D5-8+(1), n=16; D5-8+(2), n=11. T2 was taken during one menstrual cycle (C2) between D5-8+ and the remaining timepoints were taken in the other menstrual cycle (C1).

Table 3.10. Salivary and Serum Correlations

Participant	Progesterone	Estrogen
2	.46	.66
5	**.99***	.47
12	-.12	.09
18	**.89***	-.30
20	.77	.16
26	.87	.48

*p < .05. Values presented are based of serum and saliva samples obtained on the same day. One sample was taken during one menstrual cycle (C2) between D5-8+ and the remaining 4 timepoints were taken in the other menstrual cycle (C1). Only participants with the maximum 5 corresponding serum and saliva samples are presented in this table. Even though saliva was collected daily, participants missed days or gave low sample volumes. Additionally, not all participants had all 5 serum samples.

Table 3.11. Salivary and Serum Correlations

Participant	Progesterone	Estrogen
1	.85	-.62
3	na	.44‡
6	.65‡	.98
7	.64	.62
14	.52‡	.85‡
15	.86	.76†
16	**.97***	-.84
22	.62‡	.95‡
23	-.46‡	-.97‡
24	**.98***	.94

*p < .05. n=4 unless otherwise noted;†n=5, ‡n=3; na, < 3 matching serum and saliva samples. Values presented are based of serum and saliva samples obtained on the same day. Sample were taken during one menstrual cycle (C2) between D5-8+ and the remaining 4 timepoints were taken in the other menstrual cycle (C1).

Energy Availability and Salivary Hormone Relationships

A repeated measures correlation was performed to determine the relationship between energy availability, energy intake, physical activity, estrogen, progesterone, and P:E2 within T1 and T2. Energy intake, energy availability, and physical activity were measured daily for seven days in each timepoint. Hormones were analyzed every other day for 7 days in each timepoint. When hormones were used in the analysis, the corresponding value (i.e., energy intake) for each variable for the same day was selected. If hormones were not part of the analysis, all seven days in each timepoint were used.

Only energy availability and physical activity were correlated across both timepoints (p=.002, r=-0.174, 95% CI [-0.28, -0.06]) with a stronger correlation in T1 (p = .004, r= -0.293, CI

[-0.47, -0.09]) compared to T2 ($p > .05$). Additionally in T1, salivary P:E2 and energy availability demonstrated a potential moderate ($r = .277$), albeit nonsignificant ($p = .056$), relationship. Energy intake was not correlated with either estrogen or progesterone concentrations across both timepoints ($p > .05$). However, energy intake and P:E2 were moderately correlated for T1 ($p = .026$, $r = .321$, 95% CI [0.04, 0.55]) but not for T2 ($p = .44$).

A Pearson correlation assessed the relationship between energy availability, its components, and estrogen and progesterone and is displayed in Table 3.12. Lower FFM was associated with higher energy availability ($p = .001$, $r = -.672$), lower progesterone range (T1: $p = .041$, $r = .460$; T2: $p = .001$, $r = .674$), and lower T1 estrogen AUC ($p = .041$, $r = .460$). Using an independent t-test, FFM was similar when assessed in females with and without menstrual dysfunction ($p > .05$).

Higher energy availability was associated with lower RMR, FFM, and T2 progesterone range ($p = .009$, $r = -.558$; $p = .001$, $r = -.672$; $p = .032$, $r = -.479$) and higher carbohydrate intake ($p = .039$, $r = 4.54$), but no relationship emerged with fat or protein intake ($p > .05$). RMR and FFM demonstrated similar relationships, with RMR and FFM both having a positive relationship with T1 estrogen AUC ($p = .046$, $r = 4.51$; $p = .041$, $r = .460$), T1 ($p = .069$, $r = .415$; $p = .015$, $r = .537$) and T2 progesterone range ($p = .005$, $r = .605$; $p = .001$, $r = .674$) while the RMR ratio was only correlated with RMR ($p = .044$, $r = .44$).

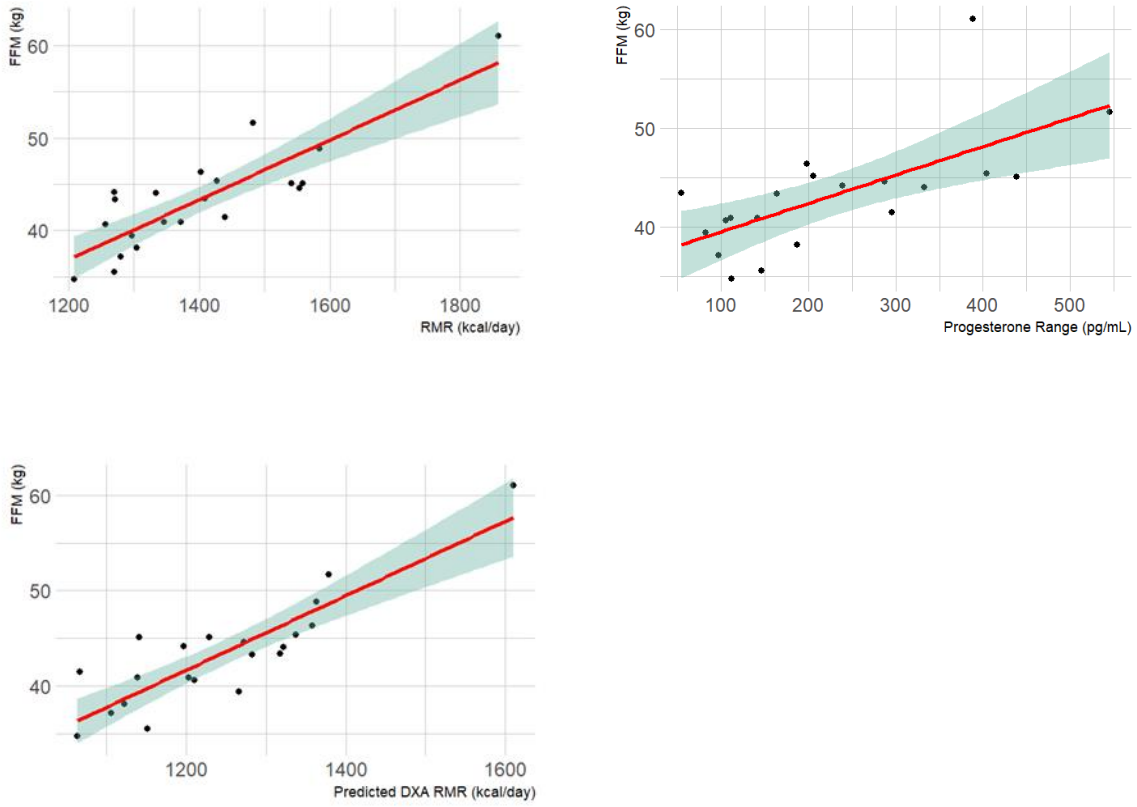
Table 3.12. Energy Availability Correlations

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. FFM	1.00																		
2. RMR	**0.86****	1.00																	
3. pRMR	**0.87****	**0.66****	1.00																
4. mRMR:pRMR	0.02	**.44***	-0.38	1.00															
5. EA	**-0.67****	**-0.56****	**-0.46***	-0.14	1.00														
6. Energy Intake	-0.11	-0.12	0.05	-0.18	**0.73****	1.00													
7. Carbohydrate	0.08	-0.03	0.20	-0.25	**0.45***	**0.79****	1.00												
8. Fat	-0.23	-0.22	-0.01	-0.24	0.30	0.13	0.01	1.00											
9. Protein	0.01	0.12	0.01	0.13	0.02	0.11	-0.26	0.14	1.00										
10. Exercise	0.03	-0.08	0.05	-0.13	-0.33	-0.06	-0.02	-0.20	0.21	1.00									
11. PA	0.34	0.27	**0.43***	-0.16	-0.13	0.25	0.23	-0.10	0.18	0.38	1.00								
12. P AUC T1	0.38	0.34	0.40	-0.05	0.05	0.33	0.23	-0.07	0.31	-0.10	0.31	1.00							
13. P Range T1	**0.54***	0.42	**0.70****	-0.31	-0.07	0.26	0.26	0.17	0.18	-0.22	0.26	**0.57****	1.00						
14. P AUC T2	0.31	0.33	0.25	0.13	0.07	0.31	0.23	0.00	0.31	-0.11	0.23	**0.80****	**0.58****	1.00					
15. P Range T2	**0.67****	**0.61****	0.43	0.25	**-0.48***	-0.14	-0.02	-0.26	-0.02	-0.05	0.32	**0.58****	0.41	**0.7****	1.00				
16. E2 AUC T1	**0.46***	**0.45***	**0.55***	-0.11	-0.12	0.17	0.07	0.02	0.33	-0.03	0.43	**0.74****	**0.56***	0.43	0.28	1.00			
17. E2 Range T1	0.03	0.14	0.03	0.12	-0.07	-0.09	-0.17	0.13	0.23	-0.16	0.02	0.05	0.27	-0.04	-0.18	0.42	1.00		
18. E2 AUC T2	0.38	0.35	0.42	-0.08	-0.11	0.07	-0.13	0.11	0.31	-0.02	**0.48***	**0.56***	**0.47***	**0.49***	0.41	**0.77****	0.31	1.00	
19. E2 Range T2	0.09	0.16	-0.05	0.21	-0.18	-0.28	-0.35	-0.23	-0.12	-0.15	0.16	0.15	-0.02	0.00	0.18	0.40	**0.60****	**0.58****	1.00
N	21	21	21	21	21	21	21	21	21	21	21	20	20	20	20	20	20	20	20

*p < .05; **p < .01; FFM, fat free mass (kg); RMR, resting metabolic rate; pRMR, DXA predicted RMR; EA, energy availability; PA, physical activity; P, progesterone; E2, estrogen; AUC, area under the curve. All energy intake is measured in kcal. All hormones are measured in pg/mL.

Linear regression was performed to determine if energy availability and hormones predicted FFM, using all significant bivariate variables from the correlation matrix above. RMR (β = .015, 95% CI [.006, .025], predicted DXA RMR (β = .022, 95% CI [.012, .032], and T2 progesterone range (β = .009, 95% CI [.001, .017) were the strongest predictors (r^2 = .929) of FFM while controlling for fitness (VO_{2peak}) (Figure 3.7).

Figure 3.7. Predictors of FFM



Note. Individual correlations of RMR, predicted DXA RMR, and Progesterone T2 range with FFM are displayed.

Relationships without Menstrual Dysfunction

A secondary analysis was performed that included only females without menstrual dysfunction. The three participants that had menstrual dysfunction during the cycle when RMR, DXA, and energy intake were measured also had menstrual dysfunction for both cycles. These participants were removed, and the data was reanalyzed. A paired t-test confirmed that no changes in significance to the previous values in demographic, energy availability, hormones, and menstrual cycle characteristics, with the exception that the progesterone minimum became significant (p=.046) when the values in T1 and T2 (116.8 ± 127.2, 184.63 ± 138.7) were compared.

A secondary Pearson correlation that mirrored the previous analyses was ran with only eumenorrheic females (Table 3.13). By removing participants with menstrual dysfunction (n = 3), the relationships changed. FFM was no longer correlated with T1 progesterone range and T2 estrogen AUC (p > .05) but had significant correlations with physical activity (p = .024, r = .528), T2 progesterone AUC (p = .044, r = .496), and T2 estrogen AUC (p = .028, r = .533). Additionally, RMR was no longer correlated with predicted RMR while energy availability became correlated with T2 progesterone AUC (p = .043, r = .496).

Table 3.13. Energy Availability Correlations with Only Eumenorrheic Females

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. FFM	1.00																		
2. RMR	**0.77****	1.00																	
3. pRMR	**0.78****	0.382†	1.00																
4. mRMR:pRMR	-0.02	**0.54***	**-0.56*‡**	1.00															
5. EA	**-0.54***	-0.41†	-0.25†	-0.16	1.00														
6. Energy Intake	0.02	0.02	0.22	-0.18	**0.76****	1.00													
7. Carbohydrate	0.20	0.11	0.39	-0.24	**0.52***	**0.83****	1.00												
8. Fat	-0.22	-0.24	0.07	-0.26	0.28	0.12	0.02	1.00											
9. Protein	-0.05	0.21	-0.02	0.21	0.08	0.14	-0.15	0.25	1.00										
10. Exercise	0.11	-0.03	0.14	-0.11	-0.42	-0.09	0.04	-0.21	0.03	1.00									
11. PA	**0.53*‡**	0.42	**.61****	-0.17	-0.18	0.24	0.28	-0.11	0.17	0.37	1.00								
12. P AUC T1	0.40	0.37	0.41	-0.05	0.15	0.36	0.30	-0.04	0.24	-0.16	0.32	1.00							
13. P Range T1	0.38†	0.20	**.62****	-0.37	0.16	0.37	0.36	0.24	0.20	-0.23	0.30	**0.57***	1.00						
14. P AUC T2	**0.50*‡**	**0.50***	0.36	0.12	0.05	0.32	0.32	-0.01	0.28	-0.17	0.21	**0.82****	**0.66****	1.00					
15. P Range T2	**0.71****	**0.63****	0.34†	0.25	-0.41†	-0.09	-0.03	-0.25	0.05	0.00	0.37	**0.60***	0.34	**0.78****	1.00				
16. E2 AUC T1	0.43†	0.42†	**.537***	-0.13	-0.01	0.22	0.18	0.05	0.25	-0.08	0.45	**0.73****	**0.53***	0.43	0.26	1.00			
17. E2 Range T1	0.11	0.27	0.09	0.12	-0.12	-0.11	-0.16	0.13	0.26	-0.19	0.00	0.05	0.32	-0.06	-0.16	0.44	1.00		
18. E2 AUC T2	**0.53*‡**	0.43	**0.52*‡**	-0.11	-0.11	0.09	-0.03	0.12	0.19	-0.10	0.47	**0.55***	**0.50***	0.46‡	0.47	**0.77****	0.30	1.00	
19. E2 Range T2	0.28	0.35	0.04	0.21	-0.29	-0.30	-0.37	-0.26	-0.12	-0.16	0.16	0.18	0.04	-0.01	0.23	0.47	**0.60***	**0.62****	1.00
N	18.00	18.00	18.00	18.00	18.00	18.00	18.00	18.00	18.00	18.00	18.00	17.00	17.00	17.00	17.00	17.00	17.00	17.00	17.00

*p < .05; **p < .01; significance gained, †significance lost compared to Table 3.12; FFM, fat free mass (kg); RMR, resting metabolic rate; pRMR, DXA predicted RMR; EA, energy availability; PA, physical activity; P, progesterone; E2, estrogen; AUC, area under the curve. All energy intake is measured in kcal. All hormones are measured in pg/mL.

The multiple step-wise linear regression results were similar to the previous results when all the participants were pooled, with RMR, DXA predicted RMR, and T2 progesterone range being the best predictors of FFM (r^2 = .928), but controlling for fitness no longer affected the results. Albeit, not significant (p = .053), T2 progesterone AUC is a potential predictor. However, when physical activity (β = -.055, 95% CI [-.105, -.005]), was controlled for instead of fitness, RMR, DXA predicted RMR, T2 progesterone range, and T2 progesterone AUC were all

significant predictors of FFM (r^2 = .957; β = .017, 95% CI [.010, .024], β = .029, 95% CI [.021, .036], β = .018, 95% CI [.010, .026], β = -.001, 95% CI [-.002, .001]).

Discussion

This study explored the relationship of energy availability, estrogen, and progesterone in physically active females. On average, the females in this study exercised ~3 hours per week, which slightly exceeds the ACSM recommendation of 150 minutes per week and is representative of a physically active population. Our findings revealed that most of the females had reduced energy availability (71%) or low energy availability (19%), while only 10% displayed adequate energy availability. This latter finding is bothersome, since it suggests that most physically active females are not properly balancing energy intake with energy expenditure. Furthermore, females in a reduced energy availability state did not follow typical patterns expected for the relationships between FFM, estrogen and progesterone. However, when only eumenorrheic females were included in the analyses, the expected patterns emerged.

Our first hypothesis stated that regardless of fitness level (VO_{2peak}), women with lower fat free mass will have lower energy availability, lower RMR ratio, lower levels of estrogen and progesterone AUC, less range in hormone levels (less differences in min to max values) and increased anovulatory cycles. However, when the relationships between energy availability and hormones were analyzed with all females in the study, FFM and RMR followed the expected positive relationship with each other as did progesterone range and T1 estrogen AUC with FFM, yet the other relationships were not as expected. For example, instead of energy availability having a positive relationship with FFM, a negative relationship emerged and no relationship was found for FFM and the RMR ratio, indicating females in a reduced energy availability state may not follow typical patterns. Koltun et. al (2020) found that oligomenorrheic/amenorrheic

exercising females with LEA had lower FFM and body fat percentage when compared to eumenorrheic controls. In contrast, when our lowest energy availability participants (n=5) were compared to our high energy availability participants (n=5), the group with the lowest energy availability had higher FFM but no difference in body fat percentage when compared the highest energy availability group. However, since the majority of our participants were only in a reduced energy availability state it is not directly comparable to FFM in LEA but these results suggest women with higher FFM are not properly balancing energy intake and exercise. Our females with the lowest energy availability had an average of 10kg more of FFM than the females with the highest energy availability yet recorded an average of ~400 kcal less per day. It is hard to discern the etiology for the decreased energy intake, as decreased energy availability can be intentional or unintentional. Energy intake may be reduced intentionally to decrease body fat percentage, to optimize performance or for social reasons, with many young females attempting to lose weight to improve appearance (Martinsen et al., 2010). Eating disorders (clinical and subclinical) are another potential cause and are hard to distinguish from athletes who are only trying to improve performance and should be evaluated by a medical professional. LEA may unintentionally be caused by suppression of appetite by prolonged exercise, as exercise stimulates anorexigenic hormones (Hackney & Constantini, 2020) or by consuming a low energy density diet (Melin et al., 2016).

Females with lower FFM had lower estrogen AUC during T1 but not T2, progesterone AUC was not associated with FFM, yet the progesterone range was significantly lower at both timepoints. Previous research has shown that decreased estrogen and progesterone are associated with decreased FFM in ovariectomized rats (Toth et al., 2001) and postmenopausal women (Poehlman, 2002), but this research does not take into account fluctuations of estrogen and progesterone across the menstrual cycle. Our study found that FFM was not associated

with progesterone AUC at either timepoint across the cycle and was only correlated with estrogen at the beginning of the cycle in the follicular phase. Even though progesterone AUC was not associated with FFM, progesterone range in both timepoints was association with lower FFM, indicating that women with lower FFM have less of a change (i.e., difference from minimum to maximum) in progesterone values per timepoint.

However, removing the three participants with menstrual dysfunction during the month that T1 and T2 were measured from the analyses, the expected positive relationship between FFM and estrogen and progesterone AUC in T2 emerged and the positive relationship with T2 progesterone range was sustained. The positive associations during T2 were expected since hormones were lowest in T1, while T2 should have captured estrogen and progesterone peaks post ovulation. These results indicate the need to distinguish between eumenorrheic women with normal cycles and those with menstrual dysfunction when measures are taken across the menstrual cycle. This aligns with the current recommendation by De Jonge et al. (2019) to use hormones to confirm menstrual function and exclude any participants with menstrual dysfunction. Without confirming hormones and menstrual function, participants may be classified into the wrong menstrual phase, which is particularly important if menstrual phase is imperative to the research question. For example, it is impossible to categorize an anovulatory cycle into follicular and luteal phase since ovulation did not occur. Since physically active females have a high prevalence of subclinical menstrual dysfunctions, it is recommended to confirm menstrual function with hormones to identify true menstrual cycle related changes.

Females with lower FFM were hypothesized to have lower energy availability, RMR, and RMR ratio. Our results indicated lower FFM was associated with lower RMR as expected, since FFM is the main driver of RMR (Blundell et al., 2020). Surprisingly, FFM was not associated with RMR ratio in either analysis, while lower FFM was associated with higher energy

availability when all participants were pooled but this relationship no longer existed when only eumenorrheic females were analyzed. Decreased RMR is an indication of an energy conservation state and is associated with LEA (De Souza et al., 2003). In previous studies, energy deficient female's RMR measured 20-40% less than expected (Benton et al., 2020; Jasienska, 2003) and a RMR ratio with a cutoff value of .94 (predicted was measured with DXA) was indicative of LEA (Strock, Koltun, Southmayd, et al., 2020). Conversely, our results showed no direct relationship with energy availability or the RMR ratio while a moderate negative relationship between energy availability and RMR occurred. The cutoff value of .94 for the RMR ratio is based off total triiodothyronine (TT_3) levels, since decreased TT_3 is a well-known biomarker associated with LEA. Most of the participants were only in a state of reduced energy availability, indicating that a RMR ratio of .94 might only be applicable for identifying females with LEA. In addition, a previous study (Koehler et al., 2016) indicated that this cutoff value may not discriminate females with LEA that have normal or subclinical menstrual dysfunction. This may also explain why the RMR ratio was not correlated with any estrogen and progesterone measurements. Interestingly, the relationship between predicted RMR and RMR was no longer significant when females with menstrual dysfunction were removed. Although we did not have a large enough sample size to directly compare, Stock et al. (2020) determined no difference between subclinical menstrual dysfunction and eumenorrheic participants when comparing DXA predicted RMR or RMR ratio, but did not investigate the relationship between the two. The negative association between energy availability and RMR could be partially explained by the decrease in FFM. FFM is the main driver of RMR, therefore less FFM should equate to a lower RMR. Females with higher energy availability had decreased FFM, accounting for the lower RMR. Furthermore, higher FFM was associated with higher physical activity. This could indicate

that females with lower FFM had higher energy availability due to less energy expenditure during physical activity.

In the current sample of physically active females, FFM was not different in females with and without menstrual dysfunction. Interestingly, even though there was not a significant difference, the females that experienced menstrual dysfunction (n=6) had higher FFM (46.3 ± 7.4 vs 42.2 ± 4.8), BMI (25.9 ± 4.2 vs 22.9), and body fat percentage (31.5 ± 10.4 vs 29.4 ± 8.5) than females without menstrual dysfunction (n=15). However, the sample size with menstrual dysfunction was low, with 15% of the cycles considered anovulatory and 8% LPD occurring in nine out of 39 menstrual cycles amongst six participants, making it difficult to compare the two groups. Research is limited but our results are in agreement with previous studies that found no difference in FFM and subclinical menstrual dysfunction (Reed et al., 2015; Schaumberg et al., 2017). Furthermore, the percentages of anovulation are similar to previously reported values (12-21%) in exercising females (De Souza et al., 1998b, 2010b), and while our percentage of LPD cycles was normal when compared to all women (3-20%), it was much lower than the previously reported prevalence of 47% of menstrual cycles in exercising females (De Souza, 2003). This difference may be due to the population used in this study. Most menstrual function studies have focused on endurance athletes whereas this study used a diverse population of athletes that were largely involved in strength training. Menstrual dysfunction should also not always be assumed to be due to energy availability and other factors associated with menstrual dysfunction, such as hyperandrogenism or polycystic ovary syndrome (PCOS) should be ruled out. Koltun et al. (2019) discovered 17% of presumed hypothalamic oligomenorrheic and amenorrheic athletes had hyperandrogenism but the prevalence is not known in an exercising population with subclinical menstrual dysfunction. Our results only identified three out of six

females at risk for low energy availability, with the remaining three in a reduced energy availability state and unexplained menstrual dysfunction.

It is hard to identify if our energy availability, energy intake, and exercise are comparable with other physically active females, due to the variance in exercising females and very few free-living studies have assessed these variables in physically active females. Nonetheless, other free-living studies have reported energy availability ranging from 35-42 kcal/kg FFM/d in females that are eumenorrheic or have subclinical menstrual dysfunction, indicating the exercising females studied in these previous investigations were in a reduced energy availability state as well (Ihalainen et al., 2021; Reed et al., 2011, 2015). Comparisons of energy availability should be done with caution, depending on the methods used to calculate energy availability. Guebels et al. (2014) calculated energy availability from exercise logs and 7-day weighed food records using four different methods: 1) all exercise, 2) all exercise + walking + bike community, 3) all exercise ≥ 4.0 METS, and 4) All exercise ≥ 4.1 METS. Energy availability was 34.0, 28.2, 34.2, and 36.7 respectively. However, the participants in this study were amenorrheic, not allowing for us to directly compare our results. However, this previous study does indicate the need to determine how energy availability is calculated in various studies. For our study, all exercise greater than 10 minutes was included and restrictions were not placed on the type of exercise, therefore walking was included. Our energy intake appeared lower compared to other studies that investigated the same population. Our average energy intake was 1675 kcal/day while others reported 1957–2340 kcal/d (Reed et al., 2011, 2015) but our participants exercised ~3 hours less per week compared to other studies, which could account for the difference in energy intake. Furthermore, the total FFM (kg) was also similar to our participants (44.3 vs 43.4) and BMI (21.1 vs 23.8) respectively but our participant's did have a larger average body fat percentage (25.2 vs 30.0). Overall, our finding appear to be similar to previous free-living

studies, indicating our calculated energy intake and energy availability are comparable with other studies focused on physically active females.

To summarize, the majority of our first hypothesis is rejected. Lower FFM was not associated with lower RMR ratio, lower energy availability, lower levels of progesterone in T1, lower levels of progesterone and estrogen in T2, and less estrogen range in either timepoint. However, lower FFM was associated with lower levels of estrogen in T1 and lower ranges of progesterone in T1 and T2. Additionally, only RMR, DXA predicted RMR and T2 progesterone range significantly predicted FFM.

Our second hypothesis stated that energy intake will have a negative correlation with estrogen concentrations, with energy intake being high when estrogen concentrations are low and this hypothesis is also rejected. Using 3-4 days of hormones and energy intake (hormones were only assessed every other day) per timepoint, a repeated measures correlation indicated no relationship with energy intake and progesterone or estrogen, but a higher P:E2 ratio was associated with lower energy intake at the beginning of the cycle. Additionally, energy intake was not correlated with estrogen and progesterone AUC or range in either timepoint. One potential reason for these findings may be due to estrogen, which acts as an appetite suppressant at high levels. After evaluating the salivary hormone profiles, 44% experienced peak estrogen values post ovulation as opposed to the expected maximum peak value that should occur prior to ovulation. Progesterone acts as an appetite stimulant in the presence of estrogen yet no optimal P:E2 ratio has been determined. Therefore, progesterone may not be able to fully function as an appetite stimulant if estrogen is higher than expected and the P:E2 ratio is decreased. It is important to understand how sex steroid hormones modulate energy intake. It is unlikely that a physically active female will see physical performance gains if a diet is adjusted to fluctuating hormones but this is necessary to understand from a health perspective.

If a female is not adjusting energy intake to accommodate physiological needs, a reduced or low energy availability state could occur. Laboratory studies have demonstrated that hormones can be disrupted after 4 days of low energy availability (Loucks et al., 1998) but less is known about long term effects of reduced energy availability.

No current research has focused on the P:E ratio and energy intake, indicating that further research is needed in physically active females. Furthermore, as stated previously, hormonal profiles experience high inter-individual variability which may not be apparent when mean values or AUC are used. AUC can vary greatly depending on the individual length of the menstrual cycle and means may not capture the variability of a cycle. Thus, investigations are warranted that focus specifically on variability in the hormonal values using person-centered approaches for analysis that can capture the nuanced differences in hormone levels across the menstrual cycle.

Furthermore, our findings also do not align with previous studies related to energy intake patterns across the menstrual cycle. Several studies have noted that energy intake can fluctuate between 100-300 kcal across the cycle, with energy intake lowest in the follicular and peaking in the luteal (Barr et al., 1995; Buffenstein et al., 1995; Rocha-Rodrigues et al., 2021). The fluctuation is attributed to the variation of estrogen and progesterone throughout the menstrual cycle. Estrogen acts as an appetite suppressant while post ovulation, high progesterone can increase appetite in the presence of estrogen and is also associated with increased RMR (Hirschberg, 2012). Our results indicated no change of energy intake or macronutrients from T1 (follicular) to T2 (~ luteal), which started 5-8 days post ovulation. Barr et al. (1995) reported ~300kcal higher energy intake in the luteal phase compared to the follicular phase. However, energy intake in this previous study was recorded at the beginning and end of different menstrual cycles, with no set criteria to normalize the data to the same timepoints. This is

important because without set days based off menses and ovulation, it is hard to compare timepoints in menstrual cycles due to the extreme variation in menstrual cycle length and inter-individual differences in menstrual cycle phase lengths. At home ovulation tests were used to confirm ovulation in our study, a procedure that is an improvement over simply using calendar counting methods to select menstrual cycle phases. However, even though these tests are accurate for identifying rises in LH, at home ovulation tests are not 100% accurate at identifying if and when ovulation actually occurred. Without using transvaginal ultrasound to confirm ovulation, it is impossible to say with absolute certainty that ovulation occurred even if an LH surge was detected by the urinary ovulation test strips and although the T2 assessment was always made 5-8 days post positive urinary ovulation test, it is not possible to determine if the T2 measure truly reflects the luteal phase without the gold standard for ovulation and multiple days of serum hormone levels to confirm luteal phase.

Our research conflicted with previous studies and did not find a change in energy intake across the menstrual cycle, which may be a potential explanation for the lack of association with estrogen and energy intake and rejection of our hypothesis. Additionally, previous studies indicating a change in energy intake across the menstrual cycle did not use an exercising population, who have lower hormone levels compared to sedentary individuals. Lower hormone levels could be a factor and warrants further investigation into an exercising female population to determine the associations between energy intake and estrogen, progesterone, and P:E2 ratio. However, if energy intake does not change across the cycle in physically active females, multiple measures of energy intake across the cycle may not be needed which would greatly decrease the burden on the participants in future research investigations.

Although the focus of this study was not to address the quality of the diet consumed by these women but it is important to note the macronutrient intake. Our results (see Table 3.4)

indicate adequate protein intake (1.2g/kg/day) which is well over the RDA recommendation for protein (0.8/g/kg/day) for the general population and still meets the 1.2-2.0g/kg/day recommendation for athletes. Carbohydrates were adequate (45%) albeit on the very low end of current dietary recommendations of 45-65% of total intake. Most athletes should take in 5-10 g/kg/day of carbohydrates, but it varies per sport and due to the large variance in activity choices of our participants, it is hard to definitively determine the adequacy of this intake for carbohydrates. Regardless, low carbohydrate intake is a concern and has been associated with LEA, overtraining, and micronutrient deficiencies (S. L. Jordan et al., 2020; Viner et al., 2015). Even though our participants were in a reduced energy state, a positive relationship between energy availability and carbohydrate still emerged. The percentage of fat intake should be noted, which was 39% of total intake in T1 and 36% of total intake in T2, with T1 fat intake exceeding the current recommended 20-35% of total intake (*Dietary Guidelines for Americans, 2020-2025*, n.d.). Thus, we can't discern whether these women were also undernourished in addition to having low or reduced energy availability (low caloric intakes compared to energy expended). However, it is important to recognize that self-report may have biased the results, since individuals are known to chronically underreport what they eat and overreport activity levels (Lissner et al., 1989). Furthermore, a meta-analysis determined an average underreporting of ~600 kcal, which is large enough to erroneously categorize energy availability. To minimize this, an app was used to help increase compliance of recording energy intake and exercise was only self-reported when the heart rate monitor was not worn. The same researcher also reviewed days with the participants if the kilocalories appeared low (i.e., < 1000 kcal/day) and all participants verified the food logs were correct. Thus, compared to studies that utilize only self-report measures, this study has reasonable validity of results for EA and suggests that most physically active women are not properly fueling for their active lifestyle.

In addition to menstrual function, other factors should be considered when interpreting results associated with menstrual cycles. The high hormone variation in T2 between participants should be investigated further. The minimum and maximum salivary progesterone ranges and AUC in T2 varied from 54-546 pg/mL and 342-3734 pg/mL respectively and indicates a need for the range and AUC to be considered as opposed to a mean value. This large inter-individual variation could be attributed to multiple factors. Females may follow the same pattern across the menstrual cycle, but there is large inter-individual variability in hormones, making it difficult to compare groups and implies that individual-level analyses may be required to properly assess the influence of menstrual cycle phases on energy availability (Figure 3.3, 3.4, 3.5). Whereas ovulation tests are a validated method to determine ovulation, only transvaginal ultrasound is the gold standard to conclude ovulation occurred. If an ovulation test incorrectly classified ovulation (i.e., positive or negative), this would have affected the start of T2 and could have resulted in a situation where we did not capture the progesterone peak during T2. Furthermore, less is known about salivary hormone profiles with exercising females, which represent free hormones only and is equivalent to 1-2% of total serum concentrations (Janse De Jonge et al., 2019).

Although direct comparison of serum estrogen and progesterone is difficult due to different methodologies and hormone variation, our serum estrogen and progesterone concentrations aligned with previous research and values were within expected ranges. A study assessing estrogen in female rowers reported serum estrogen and progesterone (29 pg/mL, 0.4 ng/mL) in the follicular and in the luteal phase (125 pg/mL, 7.0 ng/mL) respectively (Vaiksaar et al., 2011). Interestingly, our serum estrogen measurements were higher in the follicular phase (90 pg/mL) but closer in the luteal phase (146 pg/mL) while progesterone was very similar in both phases (0.9 ng/mL, 7.7 ng/mL). Conversely, Shults et al. (2011) measured serum days 1-8

post ovulation and reported peak estrogen (214 pg/mL) and progesterone (16.5 ng/mL) that were both higher than the values from this study (Shultz et al., 2011). However, a radioimmunoassay (RIA) assay was used whereas our hormones were measured with an enzyme linked immunosorbent assay (ELISA). Plus variation in the menstrual cycle and menstrual function could account for the differences. Although our saliva and serum samples fell into the range as prescribed by the manufacturer of the assays, the comparison between different manufactures and types of assays can be difficult with each identifying a different 'normal' range. Elliot-Sale et al. (2021) highlighted difference between the estrogen and progesterone serum clinical reference ranges from three different sources with a wide variance. For example, two clinical estrogen reference ranges for the luteal phase were similar (220-734 pmol/L, 205-786 pmol/L) while a third refence range had a much greater range (82-1251 pmol/L) than the previous two ranges.

Since literature is limited on salivary estrogen and progesterone across the menstrual cycle, it is important to understand the pattern of salivary hormones compared to serum hormones. Furthermore, most previous literature is based off urinary or serum hormones, making it difficult to compare to our salivary hormones. The majority of previous research that used salivary measures, quantified hormones via RIA and resulted in different quantities than our ELISA assays. Ellison et al. (1987) reported salivary peak progesterone concentrations of 120 pg/mL in runners and 178 pg/mL in controls during the luteal phase when measured with RIA compared to our salivary luteal peak progesterone of 433 pg/mL measured with an ELISA. Even though our salivary hormones followed the typical patterns (e.g., progesterone peak in the luteal phase), the expected percentage of free hormones were different than expected. Interestingly, our salivary estrogen values all fell into the predicted 1-2% of total serum concentrations but our salivary progesterone did not. Post ovulation values ranged from

between 4-6% while follicular phase values were much larger at 20-25%. Serum progesterone values are the lowest in the follicular phase yet had the highest percentage of free progesterone in saliva. Evans et al. (1986) evaluated plasma and salivary progesterone and found a higher percentage of salivary free to plasma total in the follicular phase (12.5%) compared to the luteal phase (3%). Although plasma and salivary progesterone had a high correlation, the lack of parallelism was potentially due to progesterone transferred across the saliva gland membrane was not determined by the unbound concentration in plasma (Evans, 1986).

Previous research has published correlations between saliva and serum that range from .76 to .89 for estrogen and .75 to .93 for progesterone (Ellison, 1993; Lu et al., 1999; Worthman et al., 1990). When serum and saliva from six participants at five timepoints across two menstrual cycles were examined individually, only two had significant progesterone correlations and no significant estrogen correlations emerged, although utilizing more samples may have increased the significance. Four out of the six participants had progesterone correlations between .75-.93, while one displayed a negative correlation. No estrogen serum and saliva samples were correlated within the previously published ranges of .76-.89. This is concerning since serum is the gold standard for estrogen and progesterone, but as stated previously, our salivary hormonal profiles followed the expected patterns (i.e., two estrogen peaks across the cycle, elevated luteal progesterone). Concerns have been expressed about the difficulty of measuring estrogen accurately in saliva due to the low concentrations (Arslan et al., 2022; Lu et al., 1999) but validated assays were used to analyze salivary estrogen and progesterone and we were able to detect estrogen in most of the saliva samples (97.3%).

Limitations and Future Directions

Energy availability was measured using an established formula (energy intake – exercise energy expenditure / FFM). However, measuring the components of energy intake

poses many challenges. There is the potential to have inaccurate results with any self-report method to determine energy availability, due to the risk of underreporting in food logs and low compliance from participants in recording/logging exercise or overreporting their exercise participation. Participant compliance with measurement requirements could be improved by identifying an easier method for energy availability assessment, which would allow earlier detection of low energy availability and menstrual cycle dysfunction so that serious medical conditions could be prevented.

Exercise energy expenditure was measured with a heart rate monitor and energy expended during exercise was calculated based off heart rate from the maximal exercise test (VO_{2peak}). However, some participants struggled with exercise compliance and either forgot to wear the heart rate monitor or had lower than expected exercise participation during the study. All participants stated they performed an average of 150 minutes of exercise per week on the screening form while some participants did not achieve 150 minutes in T1 and/or T2. When the heart rate monitor was not worn, METs had to be estimated based off the participants description of the exercise. Since participants were students, it is possible that unexpectedly heavy scholastic workloads may have limited exercise participation. Many college students struggle to maintain regular exercise habits during school, with exercise typically decreasing towards the end of the semester (Pope & Harvey-Berino, 2013).

Menstrual cycles have large intra- and inter-individual variability. Our study design attempted to minimize this by having T1 assessments shortly after menses started and T2 assessments after a positive urinary ovulation test. At home ovulation tests are commonly used as a validated and reliable way to determine ovulation (Gudgeon et al., 1990; Guermandi et al., 2001; Leiva et al., 2014) but it is recommended to confirm ovulation with secondary measures (Janse De Jonge et al., 2019; McGovern et al., 2004). Serum was not collected on the day of

ovulation because it would have caused undue burden to the participants given everything else being asked of them, so the LH surge could not be confirmed in serum. Serum was analyzed 5-8 days post ovulation but upon analyses of the serum and saliva hormonal profiles, it is unclear if the LH surge detected by the ovulation test always resulted in an ovulatory event. Despite that the researchers confirmed each ovulation test with a picture sent by the participant to minimize inaccurate readings, the day with the darkest line did not always match with the estrogen peak in the follicular phase or 5-8 days from the progesterone peak. Without further hormone analysis, it is difficult to say when or if ovulation occurred. To minimize this, each timepoint was used for hormone analyses instead of determining phases.

Despite the limitations listed above, this study gave an in depth look at energy availability, estrogen, and progesterone across the menstrual cycle. Each participant recorded 14 days of energy intake and exercise (seven days at each timepoint) that allowed energy availability to be assessed across the cycle. Exercise is not always consistent in intensity, duration, and number of sessions and seven days allowed that variation to be captured, therefore giving an accurate average of energy availability. Furthermore, body composition and RMR were measured at both timepoints which gave further insight into energy availability. Body composition and RMR were consistent across the both timepoints, which was necessary to evaluate since both body composition and RMR can influence energy availability and its components. This study gave a comprehensive view of estrogen and progesterone across two menstrual cycles with serum and saliva. Additionally, menstrual function was determined in both menstrual cycles.

Future investigations should focus on individual differences instead of collapsing into groups. Due to the large variation in hormones, this could prove to be a more accurate way to determine the association of energy availability and hormones. More investigations should aim

to explore the relationships between energy availability and hormones, with the classification of menstrual function. Albeit hormone assessments are costly, but using saliva is a non-invasive way to get a full hormonal profile across a menstrual cycle. Furthermore, participants with a seemingly normal menstrual cycle could have subclinical menstrual dysfunction, which follows a different hormonal pattern than a eumenorrheic cycle. Grouping eumenorrheic females with females that have menstrual dysfunction could alter the results of studies that take place across a menstrual cycle. Most research is 1) cross-sectional, which does not display the true relationship across the menstrual cycle or 2) counting days is used to assign menstrual phase and participants may end up in different parts of the luteal phase, making it hard to compare results across phases. Importantly, the advent and wide availability of wearables will continue to make menstrual cycle tracking easier and will hopefully provide insights into less invasive and less intrusive ways to accurately track the menstrual cycle, but for the foreseeable future, hormonal confirmation will still be required to accurately assess menstrual cycle function and phases.

Conclusion

In summary, most physically active females in this study were in a reduced energy availability state and did not follow typical patterns expected, except for FFM and RMR having a positive relationship. Changes across the cycle in the components of energy availability are attributed to variations in estrogen and progesterone in previous literature, but even with a significant change in hormones, energy intake and RMR remained constant in this study. When the components of energy availability and hormones were assessed, progesterone range was positively associated with FFM and RMR yet T2 progesterone range, FFM, and RMR were all negatively associated with energy availability. While energy intake did not show a positive relationship with estrogen, energy intake was correlated with P:E2, not progesterone or

estrogen alone. Exercising females have lower hormone levels which may contribute to the discrepancies and warrants further investigation as much of the current research has focused on a sedentary population. It is important to understand why exercising females have lower hormones and to identify if there are any additional health risks other than menstrual dysfunction and at what threshold these start to occur. Further studies are needed to examine the relationship of energy availability and hormones in physically active females as a group but also as individuals, due to the large inter-individual variations in hormones.

CHAPTER IV: ASSOCIATIONS OF EARLY FOLLICULAR AND POST OVULATION PROGESTERONE, ESTROGEN, AND ENERGY AVAILABILITY IN PHYSICALLY ACTIVE FEMALES

Abstract

Energy availability describes the amount of energy available for metabolic functions after exercise energy expenditure (EEE). When a negative balance emerges between energy intake and EEE over time, otherwise known as low energy availability (LEA), an energy conservation state is created where metabolic fuels are focused on life sustaining metabolic processes and are shifted from other systems such as the reproductive axis (De Souza, 2003). LEA disrupts gonadotropin-releasing hormone (GnRH) pulsatility in the hypothalamus and the subsequent release of hormones from the pituitary and the ovaries, leading to a reduction of estrogen and progesterone and ultimately, menstrual dysfunction (Mountjoy et al., 2014). Furthermore, estrogen and progesterone directly and indirectly affect energy intake, yet less is known about their effects on energy availability. OBJECTIVE: To examine the association between energy availability, estrogen, and progesterone in the follicular phase (T1) and post ovulation (T2) while controlling for the previous phase in one menstrual cycle. METHODS: Healthy, exercising females (n=21; age 21.3 ± 3.1 years) not on oral contraceptives completed measures over one menstrual cycle. Daily saliva measurements were taken to create hormonal profiles of estrogen and progesterone. Energy availability (energy intake (kcal) – EEE (kcal)/ FFM (kg)), was measured twice within one menstrual cycle, with energy intake recorded for seven days at two timepoints and exercise participation recorded with a heart rate monitor at the participant's discretion. The first timepoint (T1) started during menses between day (D) 2-4 and the second timepoint (T2) started between 5-8 days post ovulation. RESULTS: A linear regression determined that T1 estrogen significantly predicted ($r^2 = .65$) energy availability in T2 ($\beta = -.61$, p

= .02), while adjusting for T1 energy availability, but progesterone did not affect energy availability. No direct relationship was identified between T1 energy availability and either hormone in T2. A cross-lagged model demonstrated that estrogen, progesterone, and the estrogen progesterone product (E2 x P) in T1 exhibited a negative relationship with T2 energy availability (β = -.36, p = .009; β = -.37, p = .008; β = -.31, p = .029). CONCLUSIONS: These findings provide important context to the relationship between estrogen and progesterone with energy availability across the cycle. Our data suggests that lower estrogen, progesterone, and estrogen progesterone product at the beginning of the menstrual cycle are associated with higher energy availability post ovulation and hormones may have potential time-lagged influences on energy availability.

Introduction

Physical activity and exercise can have many positive health benefits, but an imbalance of energy intake and energy expenditure may negate many of these positive effects. Energy availability describes the amount of energy available for metabolic functions after exercise energy expenditure (EEE) is subtracted from energy intake, and is expressed relative to fat-free mass (FFM) (Energy availability = Energy intake (kcal) – EEE (kcal)/ FFM (kg)) (Loucks et al., 1998; Mountjoy et al., 2014). When a negative balance emerges between energy intake and EEE over time, otherwise known as low energy availability (LEA), an energy conservation state is created where metabolic fuels are focused on life sustaining metabolic processes and are shifted from other systems such as the reproductive axis (De Souza, 2003).

The menstrual cycle is a complex and highly variable physiologic process controlled by the precise integration of multiple biological systems (e.g., endocrine, reproductive) (Alvergne & Tabor, 2018; Berbic & Fraser, 2013). The menstrual cycle is considered a ‘vital sign’ for women, indicative of overall health (American Academy of Pediatrics et al., 2006; Saei Ghare Naz et al.,

2020) and represents an important, yet often ignored factor in research involving females. LEA disrupts gonadotropin-releasing hormone (GnRH) pulsatility in the hypothalamus and the subsequent release of hormones from the pituitary and the ovaries, leading to a reduction of estrogen and progesterone and ultimately, menstrual dysfunction (Mountjoy et al., 2014). Of particular interest is subclinical menstrual dysfunction (i.e., luteal phase defect, anovulation), which occurs within the framework of an outwardly 'normal' menstrual cycle but has abnormal (i.e., decreased) estrogen and progestogen compared to a eumenorrheic cycle (i.e., ovulatory). Additionally, it is widely accepted that exercising females (i.e., engaging in physical activity for health or fitness) have lower sex steroid hormones (i.e., estrogen, progesterone) (Cumming et al., 1985; De Souza et al., 1998a; Ellison et al., 1987; Fisher et al., 1986; Jasienska et al., 2006; Lehmann et al., 1993; Matthews et al., 2012; Stoddard et al., 2006) and increased subclinical menstrual dysfunction (De Souza et al., 1998b) when compared to sedentary individuals. However, exercise alone does not appear to decrease estrogen and progesterone but alters hormones as a component of energy availability with energy intake as a driving factor (Williams et al., 2001).

Energy intake is known to vary between 100-300 kcal across the menstrual cycle and this is thought to reflect, at least in part, changes in hormones across the cycle, since estrogen and progesterone directly and indirectly affect energy intake. While estrogen is considered a potential appetite suppressant, the role of progesterone is not fully elucidated. Progesterone by itself does not change eating behavior in rats but stimulates appetite when E2 is present (Hirschberg, 2012). Furthermore, estrogen acts centrally on the hypothalamus and alters neural processing of feedback signals that control eating. Mice with deleted brain estrogen receptor (Er) α experienced increased abdominal obesity that resulted from hyperphagia (i.e., increased appetite) and hypometabolism (Xu et al., 2011). Additionally, an association between the Erα

receptors and anorexia nervosa was found, indicating a potential role of estrogen and restricted eating (Versini et al., 2010). Estrogen may exhibit a bidirectional role on energy intake depending on the energy state. Previous research has indicated that when mice are in a satiated state, estrogen inhibits feeding (Dragano et al., 2020). When ovariectomized (OVX) mice (i.e., no endogenous estrogen) were treated with estrogen, hyperphagia occurred. Interestingly, when OVX mice were fasted overnight, refeeding was slower than the control mice but when treated with estrogen, refeeding was the same (Yu et al., 2020). This indicated that estrogen is required to adapt to refeeding from an energy deficient state, which may be an important factor for anorexic and postmenopausal women that are in an estrogen-deprived state.

Energy intake decreases when estrogen is high, as is the case in the follicular phase and is at the lowest level prior to ovulation, when estrogen is highest, which can result in an ~10% decrease in energy intake (Asarian & Geary, 2006). Energy intake peaks in the luteal phase (Barr et al., 1995; Buffenstein et al., 1995; Rocha-Rodrigues et al., 2021), potentially due to the energetic costs of reproduction (Jasienska, 2003) and sex hormones modulating energy intake (Asarian & Geary, 2006). Menstrual function may also influence energy intake as women with anovulatory cycles (i.e., decreased estrogen and progesterone) had unchanged energy intake across the menstrual cycle (Barr et al., 1995; Rock et al., 1996) and postmenopausal women and women with ovariectomies demonstrated increased energy intake but estrogen replacement therapy normalized energy intake (López & Tena-Sempere, 2015). Future research should look at the associations between estrogen and progesterone with menstrual dysfunction and energy intake and carefully consider potential changes in macronutrient intake, since there is no consensus in the literature regarding this latter issue (Buffenstein et al., 1995; Chappell & Hackney, 1997; Hirschberg, 2012).

Most energy availability research indicates a relationship between LEA and menstrual dysfunction (i.e., abnormal estrogen and progesterone). Yet, the research has mostly been cross-sectional. Thus, the associations of energy availability and estrogen and progesterone across the menstrual cycle have been inferred from cross-sectional findings, but little is known about how these change across a single cycle. Therefore, the purpose of this study was to examine the association between energy availability, estrogen, and progesterone in the follicular phase (T1) and post ovulation (T2) while controlling for the previous phase in a single menstrual cycle. Our primary hypotheses were: 1) energy availability in the follicular phase (T1) will predict estrogen and progesterone concentrations post ovulation (T2), 2) estrogen in the follicular phase (T1) will predict energy availability post ovulation (T2) while progesterone will not affect energy availability. A third, exploratory hypothesis stated lower energy availability will be associated with lower estrogen and progesterone concentrations across the cycle, which will be associated with greater risk of menstrual dysfunction.

Methods

Study Design

This longitudinal study took place across one menstrual cycle. Participants were required to attend seven in person visits at the Exercise Endocrinology Laboratory at the University of North Carolina at Greensboro (UNCG).

The baseline visit occurred prior to the participant's expected start date of their menstrual cycle. Participants were (1) consented, (2) received instructions on how to collect saliva and the vials for the first week, and (3) set up a Training Peaks (TP) account with instructions on how to record exercise. The TP account was also used as a study calendar (i.e., which days to record energy intake, ovulation testing), to track the menstrual cycle, and to annotate any major changes in a normal routine (i.e., illness, life or social stressor). If any major

routine changes occurred, the participant was instructed to contact the researchers and a determination would be made to end, pause, or continue with the study.

At Home Measurements. At home measurements were assessed throughout one menstrual cycle that included saliva collection, at home ovulation testing, tracking of exercise, and recording energy intake. Saliva was collected every day to measure estrogen and progesterone. Menstrual function was recorded throughout the cycle. Additionally, at home ovulation tests were done during the prescribed days (Table 4.1). Energy intake was recorded twice across the cycle for seven days each time and the timepoints are described below. Exercise was not prescribed, and participants were encouraged to continue their normal exercise routine. Therefore, exercise was conducted at the participant's discretion and recorded with an app and a heart rate monitor. Energy availability was calculated for each day energy intake was recorded in T1 and T2.

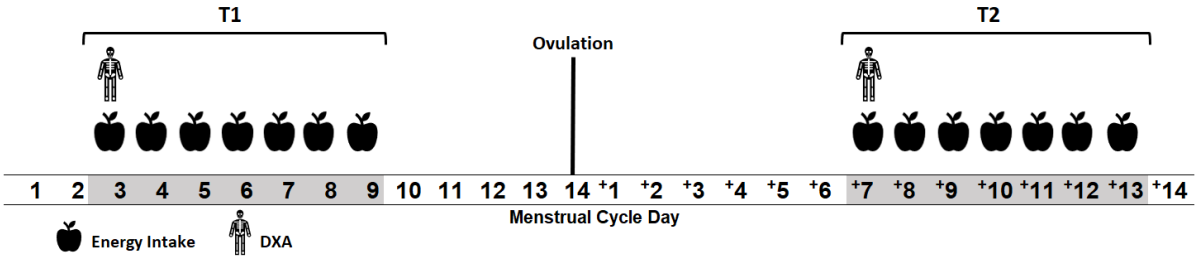
Table 4.1. Ovulation Testing Days Based on MC Length

MC Length	Testing Days
21-23	5-18
23-28	7-20
29-35	8-25
36-50	8-30

Timepoints. Two timepoints (i.e., timepoint 1 (T1) and timepoint 2 (T2)) assessed energy availability and its components and included two laboratory visits. Participants were required to fast for 12 hours prior to both visits and all visits occurred as close to waking as possible which took place between 0500-1000. Each visit marked the first day of the timepoint. T1 started in the follicular phase between D2-4 when estrogen and progesterone should be low, with estrogen starting to increase at the end of T1 while T2 started between D5-8 post ovulation when estrogen and progesterone should be high (Figure 4.2). Each visit (T1 and T2) consisted of RHR, blood pressure, resting metabolic rate (RMR) and dual energy x-ray absorptiometry

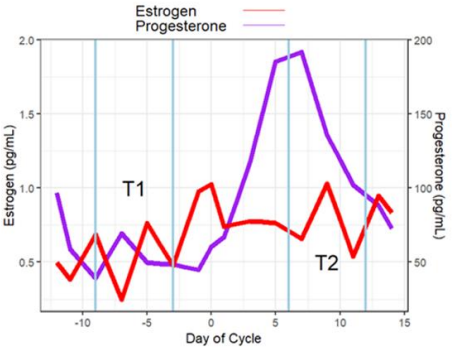
(DXA) assessments. Additionally, serum was collected at the T2 visit. At home ovulation tests determined the start of T2 (i.e., D5-8 post ovulation). If ovulation did not occur based on our criterion time frame (Table 4.1), the cycle was considered anovulatory and T2 measurements occurred no later than two days after the final ovulation test. Energy intake was recorded for 7 days during each timepoint, starting the day of the laboratory visit unless an anovulatory cycle or late ovulation occurred, then energy intake recording started prior to the visit. Energy availability was calculated for each day energy intake was recorded in T1 and T2.

Figure 4.1. Research Study Design



Note. This protocol is based off a 28-day cycle with ovulation occurring at D14. Collection days were adjusted per menstrual cycle and ovulation. Timepoint 1 (T1) started between D2-D4 in the follicular phase; Timepoint 2 (T2) started between +D5-8. D1: day of onset of menses; +: post ovulation.

Figure 4.2. An Example of the Salivary Estrogen and Progesterone Profiles Across a Single Menstrual Cycle For One Participant



Note. Progesterone AUC: T1, 325.97; T2, 853.44. Progesterone mean: T1, 52.82; T2, 142.19. Estrogen AUC: T1, 3.19; T2, 4.56. Estrogen mean: T1, 0.54; T2, 0.76. Cycle days are centered on day of ovulation (day=0). Blue lines indicate start and end of each timepoint. T1, Timepoint 1, Day 4-10 (day -9 to -3 pre ovulation on graph); T2, Timepoint 2, Day 6-12 post ovulation. Values are presented as pg/mL.

Study Procedure

Participants. Participants were recruited from UNCG via word of mouth, emails, and flyers. This study was approved by the Institutional Review Board and all subjects provided written informed consent prior to enrollment in the study. Screening procedures consisted of a modified American College of Sports Medicine (ACSM) Exercise is Medicine Health History Questionnaire and questions regarding menstrual cycle, exercise, and medical health history. Inclusion criteria were as follows: (1) have a menstrual cycle between 21-50 days, (2) age 18-35 years, (3) a minimum of 2.5 hours of exercise per week (approximately 30-45 min per day or more) and must be habitual exercisers for at least 6 months, (4) have no history of metabolic or cardiovascular diseases, eating disorders, or polycystic ovary syndrome (PCOS), (5) not take any hormonal contraceptives for 3 months prior to the start of the study or take any medications that would alter the metabolic or reproductive hormones (e.g. anxiety, depression, stimulants), (6) must not currently be using tobacco products (e.g. smoking, vaping), (7) not be actively dieting to lose weight, (8) are not or do not plan to become pregnant during the duration of the study and (9) have no internal metal (e.g. hip replacement, fixation of the spine).

Menstrual Cycle Characteristics

A menstrual cycle was defined as the first day of menses (Day 1) until the day prior to onset of the next menses. For this study, the follicular phase was defined as D1 through the day of the LH surge (i.e., indicating ovulation) regardless of length and T1 was measured during the follicular phase. The luteal phase is the day after the LH surge until the day before the onset of menses regardless of length. Luteal phase days are referred to as days post ovulation but if ovulation did not occur, phases could not be determined. T2 was expected to be measured in the luteal phase based off the at home ovulation tests, but ovulation could not be confirmed in all participants.

Participants verified having consistent menstrual cycles every 21-50 days or were asked to track one menstrual cycle prior to starting the study. The menstrual cycle length 21-50 days was selected because even though a 'normal' menstrual cycle is between 21-35 days, it is also considered normal for menses to fluctuate up to a cycle length of 45 days for the first few years after menarche (Klein et al., 2019). Additionally, this allowed participants with potentially subclinical menstrual dysfunction to participate. Menses was logged into Training Peaks for both menstrual cycles and menstrual cycle function and history was assessed once with the Low Energy Availability in Females Questionnaire (LEAF-Q) (Melin et al., 2014) prior to the first visit. Participants were instructed to contact the research team on D1 of each menstrual cycle to schedule subsequent visits.

Menstrual Cycle Classification

Ovulation testing days were adjusted based on the length of the previous self-reported menstrual cycle (Table 4.1). Ovulation testing days were selected as the highest probability of ovulation occurring within that menstrual cycle length (Soumpasis et al., 2020). Participants texted a daily picture of each ovulation test to the research team via WhatsApp and continued testing daily until the research team confirmed a positive ovulation or the number of testing days has been reached. If the participant failed to text a picture for two days, the research team contacted the participant. At home ovulation testing is proven to be accurate but ovulation was also confirmed by serum progesterone post study. Failure to receive a positive ovulation test and either a serum progesterone > 3.0ng/mL (American Society for Reproductive Medicine and the Society for Reproductive Endocrinology and Infertility, 2021) or an increase of salivary progesterone that was 2 SD above the mean of progesterone post ovulation (Ellison, 1988), resulted in the cycle being classified as anovulatory. A luteal phase deficit (LPD) was defined by < 10 days post ovulation to the start of the next menses (De Souza et al., 1998b).

Anthropometrics

Nude body mass was measured to the nearest 0.1 kg on a digital scale (WB-800S Plus; Tanita Corporation, Tokyo, Japan) at each visit while height was measured by a wall mounted stadiometer (Model216; Seca, Chio, CA) to the nearest 0.5 cm at the beginning of the study. Body mass was also calculated via the DXA analyses. Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2).

Body Composition (DXA)

Dual-energy X-ray absorptiometry (DXA) (Lunar Prodigy Advance, GE Healthcare, Madison, Wisconsin) was utilized to determine whole-body composition by a certified operator. Participants were fasted for a minimum of 2 hours prior to the DXA and a pregnancy test was administered prior to the scan to confirm that the participant was not pregnant. Each participant wore a gown and removed all metal before the scan, which was performed twice in one menstrual cycle (C2) (i.e., T1, T2) to determine if body composition changed across the menstrual cycle. The DXA scanner has a <1% coefficient of variation for body composition measurements. Body composition was calculated using enCORE 2011 software (version 13.60) by one researcher.

Energy Availability

Energy availability describes the amount of energy available for metabolic functions after exercise energy expenditure (EEE) is subtracted from energy intake, and is expressed relative to fat-free mass (FFM) (Loucks et al., 1998).

Energy availability = [Energy intake (kcal) – EEE (kcal)]/ FFM (kg)

Although there is widely accepted threshold for energy availability, the recommendation for adequate energy availability is ≥ 45 kcal/kg FFM/day whereas ≤ 30 kcal/kg FFM/day and has been recommended as the threshold for low energy availability.

Energy availability was calculated for 7 consecutive days during T1 and T2 during C2. The average FFM from T1 and T2 from the DXA analysis was used. Energy intake and exercise energy expenditure collection procedures are listed below.

Low Energy Availability in Females Questionnaire (LEAF-Q)

The Low Energy Availability in Females Questionnaire (LEAF-Q) was administered once via Qualtrics prior to the first visit. The LEAF-Q is a validated screening tool comprised of 25 questions that has a 78% sensitivity and 90% specificity. The LEAF-Q assesses risk for low energy availability (LEA) though injuries, gastrointestinal (GI) and menstrual function. Participants with a total score of ≥8 are considered at risk for LEA (Melin et al., 2014) (Appendix B).

Energy Intake

Energy intake was assessed for seven days at two timepoints (i.e., T1, T2) across one menstrual cycle. Due to subject burden and to increase compliance, only two timepoints were assessed and seven days was chosen to best evaluate the association of energy intake and exercise. Energy intake was self-logged in MyFitnessPal and began the day of each follicular and luteal phase laboratory visit, whenever possible. If the participant was anovulatory or had a late ovulation, energy intake was recorded prior to the T2 visit to allow the full seven days to be logged. Participants received detailed instructions from the researcher on how to log all food and beverages as well as a handout with portion sizes. The same researcher also confirmed MyFitnessPal entries at the next visit to ensure accuracy. MyFitnessPal is a validated smartphone app with reliable dietary analysis (Evenepoel, Clevers, Deroover, Loo, et al., 2020; Evenepoel, Clevers, Deroover, Matthys, et al., 2020; Teixeira et al., 2018). Total energy intake and macronutrient composition were calculated.

Exercise

Participants were given a Polar H10 heart rate monitor (Polar H10 Heart Rate Sensor; Polar Electro Inc., Bethpage, NY) to wear during all purposeful exercise greater than 10 minutes in duration. Purposeful exercise can include activities such as jogging, strength training, etc. but not daily living activities such as house cleaning. The heart rate monitor was linked to a recording device of the participants choice (e.g., Polar Beat, Garmin, Apple) that automatically uploaded data to the Training Peaks (TP) app. Ratings of perceived exertion (RPE) was recorded using a built-in function of TP that utilized a modified sliding scale of 0-10 for each exercise session.

Calculation of exercise energy expenditure used training heart rate divided into 7 heart rate zones equally distributed between resting heart rate (RHR) and maximum heart rate. For each heart rate zone, the metabolic equivalent (MET) was calculated using the heart rate index method ($6*(HR_{absolute}/HR_{rhr})-5$). This method has been validated and shown to be an accurate measure of VO_2 at different intensities (KANG et al., 2020; Wicks et al., 2011; Wicks & Oldridge, 2016). A MET is approximately equal to a resting value of 3.5 ml/kg/min but this varies by participant and females typically have lower values (Byrne et al., 2005). Therefore, corrected METs were calculated using the measured resting VO_2 (MET * 3.5ml/kg/min / resting VO_2 ml/kg/min). Kilocalories from exercise were quantified using the corrected METs multiplied by exercise duration (min) and weight (kg). MET values contain resting values, therefore measured resting energy expenditure (kilocalories/min) were subtracted from the exercise kilocalories to obtain the correct exercise energy expenditure (Reed et al., 2015).

If heart rate was not recorded, the participant logged a description of the activity, duration, intensity, and RPE in training peaks which was used to determine the appropriate

METs with the compendiums of physical activities (Ainsworth et al., 2011). Corrected METs was calculated prior to calculating kilocalories as described above by the same researcher.

Hormones

Blood Collection and Preparation. Participants reported to the UNCG Exercise Endocrinology Laboratory between the hours of 0500-1000 for one visit during the following ranges: D2-4, D9-11, and two consecutive days between D5-8 post ovulation then again between D5-8 post ovulation (i.e., T2) in the other menstrual cycle for a total of 5 blood samples. Participants were instructed to be fasted for 8 hours prior to arrival. Approximately 10mL of blood was collected in a serum blood collection tube. Blood samples were allowed to clot for 20 min at room temperature then centrifuged at 3000 rpm for 15 min at 4°C. The serum samples were aliquoted into multiple 2 mL polyethylene storage tubes and frozen at -80°C.

Saliva Collection and Preparation. Saliva collection was chosen to measure the daily hormonal profile for one menstrual cycle to reduce invasiveness and increase participant compliance. Measurements of E2 and progesterone in saliva have a strong correlation with serum concentrations in premenopausal women and additionally are stable at room temperature for a few days and at -20°C long-term (Bellem et al., 2011). Serum was collected to confirm the correlation between saliva and serum.

Saliva was collected every day via passive drool using polyethylene storage tubes with straws supplied to the participant by the investigator. Participants were instructed to collect their saliva immediately after waking and to refrain from brushing teeth, eating, or drinking until the sample is collected. Participants stored saliva samples in a home freezer (-20°C) until they were returned to research team at every visit, then the samples were stored at -80°C. Saliva samples were thawed at room temperature and then centrifuged for 15 minutes prior to assay.

Serum and Saliva Measurements. Estrogen (17-β estradiol) and progesterone were quantified in serum and saliva samples by enzyme immunoassay (Immuno-Biological Laboratories, Minneapolis, MN (serum); Salimetrics, Carlsbad, CA (saliva)). All hormone determinations were assayed in duplicate with all samples from a given participant on the same assay whenever possible. The sensitivity of the serum and saliva E2 assays are < 1.399 pg/mL and 0.1pg/mL and the progesterone assays have a sensitivity of 0.045 ng/mL and 5pg/mL, respectively. Samples were reanalyzed if a coefficient of variation was > 25% for saliva and > 20% for serum. The intra-assay coefficients of variation for low and high controls were 9.6% and 3.3% (saliva progesterone), 3.1% and 5.9% (saliva estrogen), and 19.2% and 5.7% (serum estrogen) respectively. Inter-assay coefficients of variation for low and high controls were 19.6% and 34.2% (saliva progesterone), 13.5% and 16.6% (saliva estrogen), and 22.9% and 22.5% (serum estrogen) respectively. Serum hormones were used to verify ovulation and salivary hormones gave a hormonal profile for each menstrual cycle.

Statistical Analysis

Statistical analyses were conducted using SPSS Statistics 24 (IBM, Armonk, NY, USA) and Mplus (version 7; Muthen & Muthen, 2012). Data was summarized as mean ± SD.

Competing linear regression models were used. Two timepoints were assessed in one menstrual cycle that consisted of seven days each time. T1 started between D2-4 while T2 started between D5-8 post ovulation. Salivary estrogen and progesterone were measured with area under the curve at each timepoint while the average of energy availability was taken at each timepoint.

A) Model 1 assessed the influence of energy availability in T1 on estrogen in T2, while controlling for estrogen in T1.

B) Model 2 assessed the influence of energy availability in T1 on progesterone in T2, while controlling for progesterone in T1.

C) Model 3 assessed the influence of progesterone in T1 on energy availability in T2 while controlling for energy availability in T1.

D) Model 4 assessed the influence of estrogen in T1 on energy availability in T2 while controlling for energy availability in T1.

A cross-lagged model was used in a secondary analysis to examine the causal influence between energy availability, estrogen, progesterone, and estrogen progesterone product (i.e., estorgen x progesterone) across two timepoints. Energy availability (X variable) was evaluated with each hormone separately (Y variable) at each timepoint. Model fit was assessed with chi-square, CFI, RMSEA, and SRMR. Autoregressive paths determined direct effects while cross-lagged paths determined direct cross-lagged effects across time. Autoregressive and cross-lagged effects were each estimated while controlling for the other.

For the exploratory hypothesis, logistic regression was used to estimate the probability of menstrual dysfunction with energy availability. Menstrual cycles were defined as normal or dysfunction (i.e., LPD, anovulation).

Results

Demographic and Reproductive Characteristics

The demographic and reproductive characteristics are shown in Table 4.2. Twenty-one participants completed the study but due to one participant having low compliance with collecting saliva, only 20 participants are used for analyses. Participants identified themselves as Caucasian (43%), Hispanic (19%), African-American (33%) and an unlisted race (5%). Weight, BMI, body fat %, or fat free mass did not change across the cycle ($p > .05$) therefore the average was taken and presented in Table 4.2. Strength training was the primary mode of

physical activity for most of the participants (81%). Most participants engaged in multiple activities, including running (57%), yoga (29%), indoor cycling (31%), soccer (19%), Zumba (14%), and dance (10%). All participants were nulliparous and underwent menarche prior to 15 years of age. Three out of 21 cycles were categorized as anovulatory (14%) while 18 menstrual cycles were confirmed as ovulatory (86%). No cycles were classified as LPD in this study.

Table 4.2. Demographic and Reproductive Characteristics for One Menstrual Cycle

	n=21
Age (years)	21 ± 3
Height (cm)	163.2 ± 4.5
Weight (kg)	63.22 ± 11.4
BMI (kg/m^2)	23.8 ± 4.0
Body fat (%)	30.0 ± 8.9
Fat free mass (kg)	43.4 ± 5.8
Age of menarche (years)	11 ± 1
Gynecological age (years)	9 ± 3
Menstrual cycle length (d)	29.6 ± 3.4
Ovulatory cycles	18
Anovulatory cycles	3

Values are mean ± SD.

Energy Availability and Hormone Characteristics

Energy availability and hormones measured in T1 and T2 are presented in Table 4.3. No changes between timepoint were significant for energy availability, energy intake, or exercise ($p>.05$). The mean represents the average value between T1 and T2. As expected, progesterone AUC and range and estrogen AUC was higher in T2 compared to T1 ($p=.001$, $p=.027$. $p=.003$).

Most participants (71%; 15/21) were in a reduced energy availability state, with 19% (4/21) classified as low energy availability (< 30 kcal/kg FFM) while only 10% (2/21) were above the recommended 45 kcal/kg FFM. Yet, the LEAF-Q classified six participants at risk (total score ≥8) for LEA by assessing injury risk, gastrointestinal function, and menstrual function. Only one participant was classified as LEA by both the < 30kcal/kg FFM value and the LEAF-Q. This participant was also the only female with menstrual dysfunction that was classified as LEA.

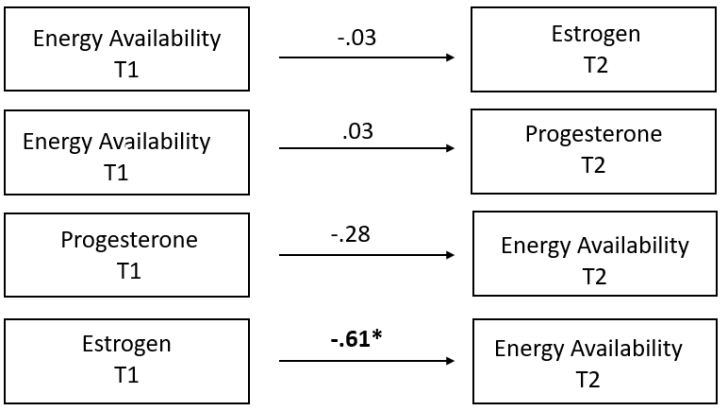
Table 4.3. Demographic, Energy Availability, and Salivary Hormone Characteristics at T1 and T2 Across One Menstrual Cycle

	T1	T2	p	Mean
EA (kcal/kg/FFM/d)	35.8 ± 11.5	35.8 ± 7.5	.993	35.8 ± 8.8
Energy Intake (kcal/d)	1682.6 ± 390.3	1667.6 ± 179.8	.855	1675.1 ± 240.1
Carbohydrate (% kcal/d)	45.0 ± 6.2	44.8 ± 7.2	.849	44.9 ± 6.2
Fat (% kcal/d)	38.5 ± 6.6	36.3 ± 7.5	.281	37.4 ± 5.4
Protein (% kcal/d)	17.4 ± 4.3	16.7 ± 5.7	.430	17.0 ± 4.7
Protein (g/kg/d)	1.2 ± 0.5	1.1 ± .37	.860	1.2 ± 0.4
Exercise duration (hour/week)	3.2 ± 1.8	2.5 ± 1.9	.190	2.9 ± 1.4
EEE (kcal/d)	168.9 ± 142.1	143 ± 152.0	.255	156.0 ± 138.3
Progesterone AUC	1197.4 ± 932.0	1765.17 ± 1033.9	**.001**	
Progesterone range	154.3 ± 109.4	226.8 ± 136.4	**.027**	
Progesterone minimum	128.4 ± 122.8	180.9 ± 137.1	.094	
Progesterone maximum	282.7 ± 174.1	407.7 ± 223.0	**.001**	
Estrogen AUC	8.3 ± 4.4	10.5 ± 4.2	**.003**	
Estrogen range	.8 ± .6	1.0 ± .7	.176	
Estrogen minimum	2.0 ± .6	1.2 ± .5	**.017**	
Estrogen maximum	1.8 ± .8	2.2 ± .9	**.004**	

Values are mean ± SD. d, day; EA, energy availability; EEE, exercise energy expenditure. *all hormones are presented as pg/mL, n=20. All other values are n=21.

Linear Regression

Figure 4.3. Linear Regressions Assessed the Influence of T1 on T2 with β values.



Note. * p < .05. Salivary hormones were used for analyses.

Model 1 assessed the influence of T1 energy availability on T2 estrogen, while controlling for T1 estrogen. Energy availability in T1 did not significantly predict estrogen in T2

while controlling for estrogen in T1 (β = -.03, p > .05). Estrogen in T1 did significantly predict estrogen in T2 (r^2 = .59, β=.73, 95% CI [.43, 1.03], p = .00).

Model 2 assessed the influence of T1 energy availability on T2 progesterone, while controlling for T1 progesterone. Energy availability in T1 did not significantly predict progesterone in T2 while controlling for progesterone in T1 (β = .03, p > .05). Progesterone in T1 did significantly predict progesterone in T2 (r^2 = .63, β = .88, 95% CI [.55, 1.22], p = .00).

Model 3 assessed the influence of T1 progesterone on T2 energy availability while controlling for T1 energy availability. Progesterone in T1 did not significantly predict energy availability in T2, while adjusting for energy availability in T1 (β = -.28, p = .09). Energy availability in T1 did predict energy availability in T2 (r^2 = .53, β = .52, 95% CI [.32, .71], p = .00).

Model 4 assessed the influence of T1 estrogen on T2 energy availability while controlling for T1 energy availability. Estrogen in T1 significantly predicted (r^2 = .65) energy availability in T2 (β = -.61, 95% CI [-1.23, -.09], p = .02), while adjusting for energy availability in T1 (β = .47, 95% CI [.28, .67], p = .00), indicating greater estrogen in T1 is associated with less energy availability in T2.

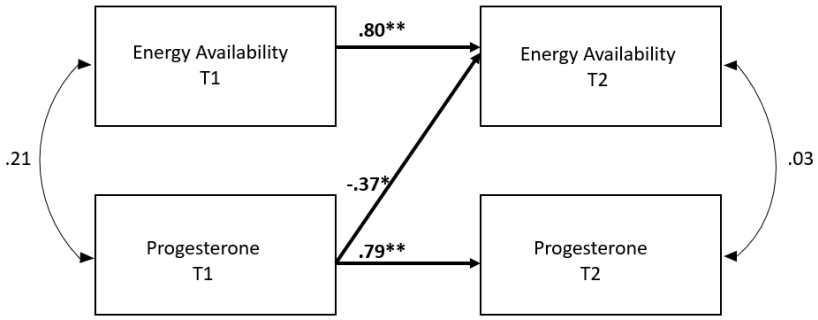
Cross-Lagged Model

The cross-lagged model with progesterone and energy availability in T1 and T2 (see Figure 4.4 for standardized estimates) demonstrated excellent model fit, $\chi^2(0) = 0.00$, p = .00, CFI= 1.00, RMSEA = .00 [CI .00, .00], SRMR = .00, and the chi-square difference test revealed that the cross-lagged model improved fit over the baseline model $\Delta\chi^2(5) = 41.51$ p = .00. All autoregressive stability paths were positive and significantly different than zero. Energy availability and progesterone both showed high stability across the cycle but did not have any significant within-time correlations. The cross-lagged paths revealed that greater progesterone at T1 was associated with less energy availability at T2 (β = -.37, p =.008).

The cross-lagged model with estrogen and energy availability in T1 and T2 (see Figure 4.5 for standardized estimates) also demonstrated excellent model fit, $\chi^2(0) = 0.00$, $p = .00$, CFI= 1.00, RMSEA = .00 [CI .00, .00], SRMR = .00, and the chi-square difference test revealed that the cross-lagged model improved fit over the baseline model $\Delta\chi^2(5) = 39.25$, $p = .00$. All autoregressive stability paths were positive and significantly different than zero. Energy availability and estrogen both showed high stability across the cycle but did not have any significant within-time correlations. The cross-lagged paths revealed that greater estrogen at T1 was associated with less energy availability at T2 ($\beta = -.36$, $p = .009$).

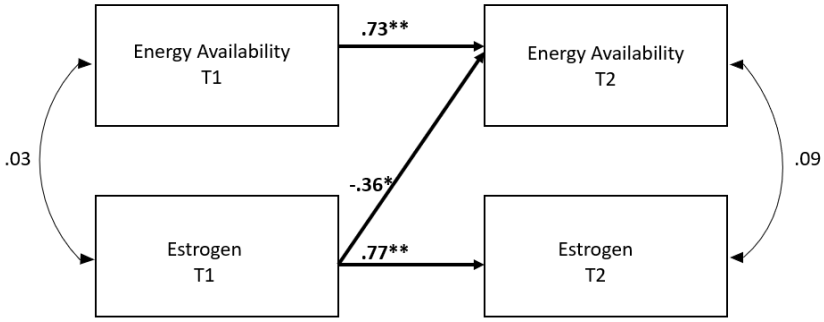
An additional analysis evaluated the product of estrogen and progesterone in the cross-lagged model with energy availability (see Figure 4.6 for standardized estimates). The product was calculated by multiplying the area under the curve of estrogen and progesterone in each timepoint. This cross-lagged model followed the same trend as the previous two, which examined estrogen and progesterone separately. This model demonstrated excellent model fit, $\chi^2(0) = 0.00$, $p = .00$, CFI= 1.00, RMSEA = .00 [CI .00, .00], SRMR = .00, and the chi-square difference test revealed that the cross-lagged model improved fit over the baseline model $\Delta\chi^2(5) = 43.640$, $p = .00$. All autoregressive stability paths were positive and significantly different than zero. Energy availability and estrogen progesterone product both showed high stability across the cycle but did not have any significant within-time correlations. The cross-lagged paths revealed that greater estrogen progesterone product at T1 was associated with less energy availability at T2 ($\beta = -.31$, $p = .029$).

Figure 4.4. Cross-lagged Model with Energy Availability and Progesterone (standardized estimates)



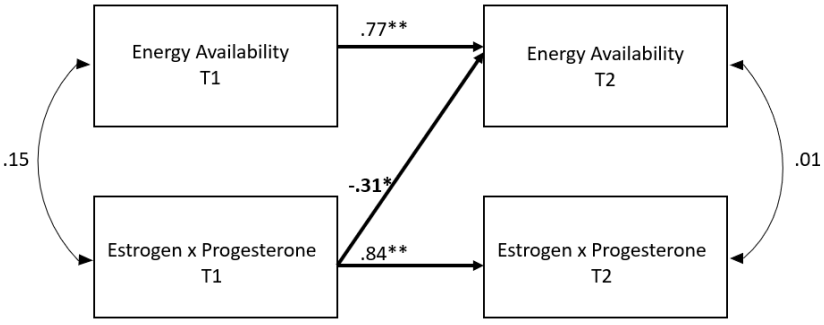
Note. Weighted lines indicated significant cross-lagged paths. Model fit: $\chi^2(0, n=20) = 0.00$, p = .00, comparative fit index (CFI) = 1.00, root mean square error of approximation (RMSEA) = .00 [CI .00, .00], SRMR = .00. p > .05; **p < .01.

Figure 4.5. Cross-lagged Model with Energy Availability and Estrogen (standardized estimates)



Note. Weighted lines indicated significant cross-lagged paths. Model fit: $\chi^2(0, n=20) = 0.00$, p = .00, comparative fit index (CFI) = 1.00, root mean square error of approximation (RMSEA) = .00 [CI .00, .00], SRMR = .00. * p > .05; **p < .01.

Figure 4.6. Cross-lagged Model with Energy Availability and Estrogen Progesterone Product (standardized estimates)



Note. Weighted lines indicated significant cross-lagged paths. Model fit: $\chi^2(0, n=20) = 0.00$, p = .00, comparative fit index (CFI) = 1.00, root mean square error of approximation (RMSEA) = .00 [CI .00, .00], SRMR = .00. * p > .05; **p < .01.

Logistic Regression

Logistic regression was performed as part of an exploratory hypothesis to determine if lower energy availability, estrogen, and progesterone was associated with menstrual dysfunction. A separate logistic regression was performed for T1 and T2 and per hormone (e.g., T1 energy availability and T1 estrogen then T1 energy availability and T1 progesterone were analyzed as separate models). Out of 20 participants, only 3 were classified as having menstrual dysfunction. No models were significant compared to the null models ($p > .05$) and energy availability, estrogen, and progesterone did not significantly predict menstrual dysfunction ($p > .05$) in any of the models. No significant relationship between energy availability, estrogen, and progesterone emerged in either the eumenorrheic or menstrual dysfunction group when assessed with a Pearson correlation. This hypothesis was exploratory due to the potential of a small number of participants having menstrual dysfunction, which could only be determined after confirmation with hormones once the study was completed.

Discussion

This study examined the direct and transactional (i.e., bidirectional) relationship of energy availability with estrogen and progesterone in physically active females. In this study we used a novel approach (i.e., cross-lagged analyses) to examine the direct and transactional relationship of energy availability with estrogen, progesterone, and the estrogen progesterone product in physically active females. Although this study has a small N and our results should be interpreted with caution, these cross-lagged analyses suggest that estrogen and progesterone in T1 were negatively associated with T2 energy availability, indicating that higher hormones in T1 are associated with lower energy availability in T2.

We hypothesized that energy availability in T1 will predict estrogen and progesterone in T2, while controlling for each hormone in T1 and our first hypothesis is rejected (Model 1 and 2).

Contrary to our expectations based on previous literature that indicated LEA resulted in disrupted estrogen and progesterone (De Souza et al., 2007; Williams et al., 2015), no direct relationship was found between T1 energy availability and either hormone in T2. Although previous research has investigated the relationship of LEA with lower estrogen and progesterone by proxy of menstrual dysfunction, direct associations with varying levels of energy availability with estrogen and progesterone across the cycle are less clear. It is well documented that strenuous exercise at any point of the menstrual cycle (Williams et al., 1999) and short term low energy availability (Loucks et al., 1998; Loucks & Thuma, 2003) can affect the hypothalamic-pituitary-gonadal (HPG) axis but neither of these previous studies looked at the direct relationship of energy availability and hormones across a cycle. Also, the studies took place in lab-controlled settings with extreme interventions using sedentary women as participants, who also had higher concentrations of estrogen and progesterone when enrolled into the study. Therefore, sedentary females may experience a larger disruption in the HPG axis with decreased energy availability than regularly exercising females or females with LEA. Since most of the research is based on laboratory-based interventions with highly controlled exercise, the effect of energy availability on estrogen and progesterone in physically active females across the cycle in free-living environments has not been studied.

In addition, it is possible that we rejected this hypothesis because of the state of energy availability in our participants. Most previous research has focused on LEA while our participants were primarily in a reduced energy availability state (71%), with only 19% classified as low energy availability (<30kcal/kg FFM/d) and only a small number (10%) displaying adequate energy availability (>45kcal/kg FFM/d). Hence, a reduced energy availability state may not be severe enough to induce a notable decrease in estrogen and progesterone in all physically active women. In a previous study, energy availability ranged between 25.8-61.8

kcal/kg FFM/d in 13 eumenorrheic exercising females with an average energy availability of 42.1 kcal/kg FFM/d (Reed et al., 2011). Our range of energy availability is similar to Reed et al. but energy availability categories were not listed so we are unable to compare prevalence of LEA. However, when 16 female long distance runners (i.e., ≥ 45 km/week) that were recruited from a local running club were evaluated, many runners were below 30 kcal/kg FFM/d but were able to maintain an ovulatory menstrual cycle (Schaal et al., 2021). Male and female professional and competitive amateur cyclist had energy availability assessed and all 10 participants maintained LEA across the cycling season (Viner et al., 2015). Our participants were in a higher energy availability state than the latter two studies but that may be due to the type of exercise, with both previous studies investigating endurance athletes while our participants were primarily strength training athletes. Although the prevalence of LEA or reduced energy availability is unknown, previous research suggests that it is prevalent amongst exercising females.

As hypothesized, T1 estrogen predicted T2 energy availability while progesterone did not affect energy availability and we accept our second hypothesis (Model 3 and 4). Interestingly, a direct relationship was found between T1 estrogen and T2 energy availability but the relationship was negative, not a positive relationship as we predicted based off previous literature that low energy availability is associated with decreased estrogen and progesterone (De Souza et al., 2007). Further analyses with a cross-lagged model indicated T1 estrogen and progesterone had a negative relationship with T2 energy availability, indicating that higher hormones were associated with lower energy availability. Lieberman et al. (2019) did a secondary analysis looking at the relationship of energy availability across three cycles in 35 previously untrained women. Energy availability groups were assigned through a controlled diet and exercise intervention and were classified as low (23.4-34.1 kcal/kg/ FFM/d), moderate

(34.9-40.7 kcal/kg/ FFM/d), and high (41.2-50.1kcal/kg/ FFM/d). The LEA group had higher follicular estrone-1-glucoronide (E1G; urinary metabolite of estrogen) compared to the moderate energy availability group and greater luteal E1G when compared to the high energy availability group. The exercise and diet intervention had a suppressive effect on E1G and pregnanediol glucuronide (PdG; urinary metabolite of progesterone) across the three menstrual cycles but was not dependent on energy availability. Although this study did not look at the direct relationship with energy availability and hormones, the LEA group had higher mean follicular and luteal phase estrogen, but not progesterone, compared to groups with higher energy availability.

The cross-lagged model was used to assess the bidirectional influence of energy availability at different timepoints across the cycle, which gives more insight than the one direction approach of linear regression. The reason that only T1 hormones are associated with T2 energy availability but not T1 energy availability on T2 hormones is unclear. Using a cross-lagged model to investigate the relationship of hormones and energy availability at different timepoints across the cycle is a novel approach in this area of research and thus, we have no previous literature for comparison. Progesterone is typically low in the follicular phase (T1) and therefore was not expected to influence energy availability while estrogen may exhibit a rise at the end of T1. Estrogen has been viewed as an appetite suppressant in the absence of high progesterone levels and for this reason, higher estrogen was expected to decrease energy intake, which in turn could decrease energy availability. Nonetheless, the associations between energy intake and hormones have only been assessed at a specific timepoint and although rapid changes (i.e., less than 24 hours) in appetite have been displayed due to addition of exogenous estrogen in OVX mice, less is known about this effect in humans and if there are time-delayed responses due to the changes in endogenous estrogen. High estrogen at the

beginning of the cycle may have lasting appetite suppressing effects at other timepoints in the cycle. Thus, a potential time-lagged response to hormones may explain why higher T1 hormones are associated with lower T2 energy availability. Furthermore, exercise is a component of energy availability, and it is well known that exercising females have lower hormones than sedentary females although the exact mechanism of the decreased hormones is unclear. Exercise can affect energy availability directly and indirectly, with exercise energy expenditure subtracted from energy intake, but exercise can also drive energy intake. Energy intake and exercise were not significantly different at either timepoint, but exercise did decrease from 3.2 to 2.5 hours from T1 to T2. The decrease in exercise could potentially influence energy availability and estrogen and progesterone. Additionally, low energy availability is theorized to drive hormone concentrations. Our participants were primarily in a reduced energy availability state and this could contribute to the contrasting findings. Hormones could potentially be a driver of energy availability in an adequate or reduced energy availability state compared to energy availability driving hormones in a LEA state. However, our sample size was small. Our results should be interpreted with caution and further investigations with a larger sample size should be conducted.

In a previous analysis (Chapter III), no within timepoint relationship between estrogen, energy intake or energy availability was discovered while there was a moderate correlation with progesterone to estrogen ratio (P:E2) and energy intake within T1. Therefore, a closer look at the effects of the P:E2 ratio should be examined, instead of each hormone individually. Estrogen and progesterone not only have independent functions throughout the body, but also have functions that are dependent on both progesterone and estrogen concentrations (Giersch et al., 2021). For example, progesterone acts as an appetite stimulate only in the presence of estrogen. Since menstrual cycles typically follow the same pattern but display large variability on

the hormone concentrations across the cycle, looking at the P:E2 ratio may give more insight into the relationship with energy availability. Thus, energy availability may be altered by the P:E2 ratio and warrants further investigation.

A third, exploratory hypothesis stated lower energy availability would be associated with lower estrogen and progesterone concentrations, which will be associated with greater risk of menstrual dysfunction. Using logistic regression, no significant relationships between hormones, energy availability, and menstrual dysfunction were found and therefore, our hypothesis was rejected. Furthermore, no correlation was determined between energy availability, estrogen, and progesterone in either the eumenorrheic or menstrual dysfunction group. This was an exploratory analysis since the potential for a small number of participants having menstrual dysfunction was recognized and perceived as likely. However, the accuracy of this perception could only be determined once the study was completed and serum and salivary hormones were analyzed. The lack of significance is most likely attributed to the small sample size of females with menstrual dysfunction (n=3) compared to females without menstrual dysfunction (n=17) and the uneven group size. Yet, previous research indicated that the magnitude of energy deficiency was associated with the frequency of menstrual dysfunction (Williams et al., 2015) but this was based off sedentary females that participated in a diet and exercise intervention that limited energy availability. In contrast, a free-living study indicated that energy availability could discriminate clinical menstrual dysfunction (e.g., amenorrhea) but not subclinical menstrual dysfunction in exercising females over three menstrual cycles (Reed et al., 2015). Amenorrheic participants averaged less energy availability (30 kcal/kg/LBM/d) compared to anovulatory (40 kcal/kg/LBM/d), inconsistently anovulatory (37 kcal/kg/LBM/d) and ovulatory (36 kcal/kg/LBM/d) exercising females (Reed et al., 2015). Interestingly, ovulatory exercising females had lower energy availability, lower energy intake, and higher energy expenditure than

anovulatory females. This highlights the need to investigate other factors associated with anovulation, such as hyperandrogenism or polycystic ovary syndrome (PCOS). Koltun et al. (2019) discovered 17% of presumed hypothalamic oligomenorrheic and amenorrheic athletes had hyperandrogenism. Even though our mean energy availability (36 kcal/kg FFM/d) and results concur with Reed et al (2015), an exact comparison of energy availability should be done with caution since their analysis used LBM instead of FFM.

Limitations and Future Directions

Menstrual cycles have large intra- and inter-individual variability. Our study design attempted to minimize this by aligning timepoints with menses and ovulation. T1 started shortly after menses while T2 began 5-8 days after a positive urinary ovulation test. At home ovulation tests are commonly used as a validated and reliable way to determine ovulation (Gudgeon et al., 1990; Guermandi et al., 2001; Leiva et al., 2014) but it is recommended to confirm ovulation with secondary measures (Janse De Jonge et al., 2019; McGovern et al., 2004). As outlined in the previous manuscript, upon serum and saliva hormonal profiles, it was unclear if the ovulation test was always correct. Transvaginal ultrasound is the gold standard for determining ovulation, but it is invasive to the patient. In addition to the ovulation test, 3-5 days of serial serum sampling is recommended to account for the variation in cycles and to determine menstrual function (Schliep et al., 2014; Wideman et al., 2013) but again, this is invasive and places a burden on the participant to have 3-5 additional laboratory visits. Daily morning saliva samples are a noninvasive way to collect multiple days of hormones but there are currently no universally accepted criterion for menstrual function in saliva. Coupling ovulation tests with other noninvasive measures may assist in determining menstrual phase and function. Wearables that can measure skin temperature, heart rate variability, heart rate, and breathing rate can help predict ovulation (Goodale et al., 2019) but wearables add extra cost to research. The current

recommendations to determine ovulation are either invasive, have high participant burden, or are cost prohibitive, indicating an urgent need to develop an accurate and easily accessible way to precisely identify ovulation.

Despite the limitations listed above, this study investigated energy availability, estrogen, and progesterone across the menstrual cycle with an in depth, novel approach. Most of the current research focuses on cross sectional data while this longitudinal study investigated two seven-day timepoints across the cycle. Each participant recorded 14 days of energy intake and exercise (seven days at each timepoint) that allowed energy availability to be assessed across the cycle. Exercise is not always consistent in intensity, duration, and number of sessions and seven days allowed that variation to be captured, therefore giving an accurate average of energy availability.

Future investigations should focus on individual differences instead of collapsing into one group. Due to the large variation in hormones, this could prove to be a more accurate way to determine the association of energy availability and hormones. Furthermore, since the T1 estrogen progesterone product AUC had a negative association with T2 energy availability, investigating the daily P:E2 ratio could give more insight on the individual differences as well. A cross-lagged model with additional timepoints should be used to determine the changes within and across timepoints as well.

Multiple menstrual cycles should be assessed, allowing T2 in one menstrual cycle to be compared to T1 in the next menstrual cycle. This would determine if there are relationships across cycles instead of only investigating within a cycle. Due to the burden on the participant, only two timepoints were used and captured the early follicular phase when hormones should be low and post ovulation when estrogen and progesterone are expected to be high. By adding a timepoint of energy availability right before ovulation, the effects of high estrogen without

progesterone could be studied. There is the potential that the start of the rise in estrogen was recorded in T1 in some participants, yet participants with longer cycles and later ovulations would not have captured the rise in estrogen in T1. Due to high variation in ovulation days between participants, estimating the time right before ovulation is difficult. To best accommodate this, ovulation should be determined in the cycle prior to data collection to best estimate ovulation in the current cycle.

Future studies should also include females using hormonal contraceptives, with an estimated 50% of female athletes using hormonal contraceptives (Martin et al., 2018). Contraceptives lower endogenous estrogen concentrations and alters the HPG axis, creating a unique estrogen and progesterone hormonal profile for each type of contraceptive (i.e., monophasic versus triphasic) that differ from eumenorrheic women (Elliott-Sale et al., 2020). Estrogen and progesterone have been associated with fluctuations in energy intake yet limited research has been performed to specifically address the effect of contraceptives on energy intake. Only five studies to date have investigated this relationship, four studies indicated that contraceptives do not affect energy intake but it is less clear if macronutrient intakes are affected (Metz et al., 2022). Contraceptive use may mask the symptoms of LEA (i.e., menstrual dysfunction) however little is known about this population and energy availability. In a small sample size of active women, with 15 not taking contraceptives and 9 taking monophasic contraceptives, estrogen and progesterone were similar during menses but the contraceptive users had lower hormones in the rest of the follicular and luteal phase. No change across the menstrual cycle was noted for energy availability in either group except the contraceptive users had significantly higher energy availability in the luteal phase during the inactive phase (i.e., placebo, no pill) compared to non-contraceptive users (Ihalainen et al., 2021). During the

inactive phase, no exogenous hormones are taken, allowing endogenous estrogen to increase which could account for the change in energy availability.

Conclusion

In summary, estrogen, progesterone, and the estrogen progesterone product in T1 exhibited a negative relationship with T2 energy availability in physically active females in one menstrual cycle. Although the relationship was unexpected and the results should be interpreted with caution due to the small sample size, this data suggests that higher estrogen and progesterone at the beginning of the menstrual cycle are associated with lower energy availability post ovulation and hormones may have potential time-lagged influences on energy availability. Further research to understand this relationship in females in reduced energy availability states is warranted. By investigating energy availability at multiple timepoints across the menstrual cycle, to include individual analysis, this could elucidate the relationship between hormones at the start of the menstrual cycle with energy availability post ovulation.

CHAPTER V: THE INFLUENCE OF STRESS AND RECOVERY ON THE ESTROGEN, PROGESTERONE, AND ENERGY AVAILABILITY RELATIONSHIP ACROSS THE MENSTRUAL CYCLE IN PHYSICALLY ACTIVE FEMALES.

Abstract

Physical activity and exercise can have many positive health benefits, but proper energy intake and adequate recovery are necessary to maintain a proper balance (i.e., adequate energy availability). Low energy availability (LEA) occurs by disrupting the balance between energy intake and EEE over time, causing an energy conservation state to be created which can result in decreased estrogen and progesterone. Furthermore, physiological and psychosocial stressors can disrupt the GnRH pulse generator and subsequently alter other hormones (e.g., estrogen, progesterone). OBJECTIVE: To examine if stress and recovery moderate the relationship between energy availability and estrogen and progesterone. METHODS: Healthy, exercising females (n=21; age 21.3 ± 3.1 years) not on oral contraceptives completed measures over one menstrual cycle. Daily saliva measurements were taken to create hormonal profiles of estrogen and progesterone. Energy availability was measured twice within one menstrual cycle, with energy intake recorded for seven days at two timepoints and exercise participation recorded with a heart rate monitor at the participant's discretion. The first timepoint (T1) started during menses between day (D) 2-4 and the second timepoint (T2) started between 5-8 days post ovulation. A laboratory visit occurred on the first day of each timepoint, where body composition was measured. The Acute Recovery and Stress Scale (ARSS) was administered four times, once at each laboratory visit and then seven days later (2 times during T1 and 2 times during T2). RESULTS: A repeated measures ANOVA revealed that the stress subscale, negative emotional state, was highest at the end of T2 compared to measurements at the beginning of T1 and T2 ($F(3, 54) = 7.07$, $p = .000$). Total stress scores were inversely

associated with estrogen (r = -.521, p=.018) and progesterone AUC (r = -.478, p=.033) in T1 but not T2. No models assessing the influence of stress and recovery on the relationship of energy availability and hormones were significantly different from zero and no significant interactions with stress or recovery were noted (p >.05) at T1 or T2 with either hormone. CONCLUSIONS: Stress and recovery do not moderate the relationship between hormones and energy availability within a timepoint or across timepoints. Negative emotional state was significantly higher post ovulation towards the end of the cycle while recovery and other stress scales remained constant throughout the cycle.

Introduction

Physical activity and exercise can have many positive health benefits, but proper energy intake and adequate recovery are necessary to maintain a proper balance (i.e., adequate energy availability). Energy availability describes the amount of energy available for metabolic functions after exercise energy expenditure (EEE) is subtracted from energy intake, and is expressed relative to fat-free mass (FFM) (Energy availability = Energy intake (kcal) – EEE (kcal)/ FFM (kg)) (Loucks et al., 1998; Mountjoy et al., 2014). Low energy availability (LEA) occurs by disrupting the balance between energy intake and EEE over time, causing an energy conservation state to be created. Metabolic fuels are then focused on life sustaining metabolic processes and diverted away from other systems such as the reproductive axis (De Souza, 2003). LEA disrupts gonadotropin-releasing hormone (GnRH) pulsatility in the hypothalamus and the subsequent release of hormones from the pituitary and the ovaries (i.e., estrogen, progesterone), leading to menstrual dysfunctions (Mountjoy et al., 2014). Dysfunction of the menstrual cycle occurs on a spectrum, ranging from the least severe perturbation (i.e., luteal phase defect (LPD)) to the most severe dysfunction, amenorrhea and all dysfunctions along the continuum present with abnormal estrogen and progesterone concentrations (De Souza, 2003).

Exercise stress with proper recovery is required to stimulate adaptations from exercise and the amount of stress required to produce adaptations varies amongst individuals. However, recovery is multi-faceted and can be affected by quality and quantity of energy intake and sleep, psychosocial stressors (e.g., academic demands), or illness (Cadegiani & Kater, 2017). When excessive stress or inadequate recovery occurs, individuals may experience a physiological or psychological imbalance that results in deleterious conditions and decreased exercise performance (Kellmann & Kolling, 2019). When recovery is not balanced with daily stress, exercise stress, energy intake, etc., an under recovered state of varying degrees (e.g., functional overreaching, nonfunctional overreaching, over training) may be created. These states are created when athletes chronically fail to allow for adequate recovery from training and life stress. Functional overreaching is a state that allows for quick recovery (i.e., days) whereas over training may take months to recover from. Individuals in an under recovered state have demonstrated similar symptoms (e.g., decreased E2, progesterone, T_3) to LEA (Stellingwerff et al., 2021), yet energy availability and menstrual function are rarely assessed in these states.

The hypothalamic-pituitary-adrenal (HPA) axis is well studied in under recovered and high stress states but not the hypothalamic-pituitary-gonadal (HPG) axis, even though the axes appear to be intertwined. Moreover, most of the evidence of the dynamic interactions of the HPA and HPG come from animal studies (Zavala et al., 2020). Nonetheless, data indicates that physiological and psychosocial stressors can disrupt the GnRH pulse generator. Glucocorticoids released during a stress response may inhibit the LH surge. In rats that were physically restrained, the LH surge was blocked in over half of the mice and resulted in decreased ovulation rates (Phumsatitpong et al., 2021). Saketos et al. (1993) demonstrated in human females that cortisol can slow LH pulse frequency through GnRH disruption, indicating a role of cortisol in menstrual disturbances (Saketos et al., 1993). Furthermore, high levels of cortisol

have been associated with amenorrhea in athletes (Ding et al., 2020). Therefore, future research is indicated to examine the relationship between cortisol and stress-recovery states with energy availability across the menstrual cycle.

Exercise stress and recovery should be measured objectively as well as subjectively. Algorithms can estimate exercise stress based off heart rate or power. Training Peaks is an online platform that allows exercise to be synced from various devices and apps and calculates a training stress score (TSS) for each exercise session. TSS is an estimate of the training load based off intensity and duration and a higher TSS has been shown to be associated with a higher rate of perceived exertion (RPE) (Alfonso & Capdevila, 2022). Furthermore, TSS can be used to determine how much recovery may be needed after an exercise session and has also demonstrated a strong dose-response relationship for changes in aerobic fitness (Sanders et al., 2017).

However, external training load alone is not sufficient to determine stress and recovery due to athletes' individualistic response to training loads. Exercise is an energy-demanding activity; therefore, it is imperative to maintain a balanced stress-recovery state for optimal health and performance. Surveys are a noninvasive diagnostic tool to identify the athlete's stress-recovery state (Kölling et al., 2015) and can investigate if an athlete is getting proper recovery from stress, which could be from exercise or other life-stressors (e.g., family, work). Validated surveys such as the Acute Recovery and Stress Scale (ARSS) use a multidimensional approach by assessing the current stress-recovery state at an emotional, mental, physical, and overall level (Kellmann & Kolling, 2019). The ARSS assesses acute recovery and stress in athletes and identifies how the athlete is feeling that day, making it useful to identify the general trend of an athletes recovery and stress (Kölling et al., 2015, 2020).

Energy availability is influenced by energy intake, EEE, and FFM. However, less is known about secondary factors such as stress and recovery that may influence the relationship between energy availability and hormones across the menstrual cycle. Therefore, the purpose of this study was to examine stress and recovery across the menstrual cycle and to determine if stress and recovery moderate the relationship between energy availability and estrogen and progesterone. Our hypothesis was that energy availability will predict menstrual cycle hormone concentrations, but this relationship will be stronger with increased recovery and decreased stress scores.

Methods

Study Design

This longitudinal study took place across one menstrual cycle. Participants were required to attend seven in person visits at the Exercise Endocrinology Laboratory at the University of North Carolina at Greensboro (UNCG).

The baseline visit occurred prior to the participant's expected start date of their menstrual cycle. Participants were (1) consented, (2) received instructions on how to collect saliva and the vials for the first week, and (3) set up a Training Peaks (TP) account with instructions on how to record exercise. The TP account was also used as a study calendar (i.e., which days to record energy intake, ovulation testing), to track the menstrual cycle, and to annotate any major changes in a normal routine (i.e., illness, life or social stressor). If any major routine changes occurred, the participant was instructed to contact the researchers and a determination would be made to end, pause, or continue with the study.

At Home Measurements. At home measurements were assessed throughout one menstrual cycle that included saliva collection, at home ovulation testing, tracking of exercise, and recording energy intake. Saliva was collected every day to measure estrogen and

progesterone. Menstrual function was recorded throughout the cycle. Additionally, at home ovulation tests were done during the prescribed days (Table 5.1). Energy intake was recorded twice across the cycle for seven days each time and the timepoints are described below. Exercise was not prescribed, and participants were encouraged to continue their normal exercise routine. Therefore, exercise was conducted at the participant's discretion and recorded with an app and a heart rate monitor. Energy availability was calculated for each day energy intake was recorded in T1 and T2. The ARSS was sent via WhatsApp at the last day of each timepoint.

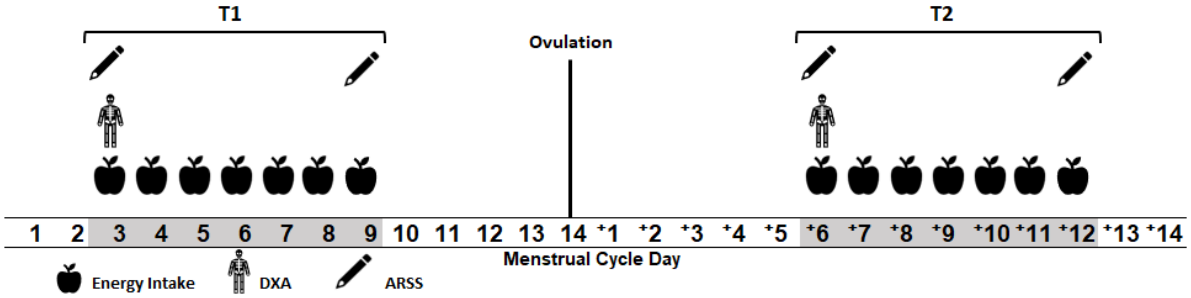
Table 5.1. Ovulation Testing Days Based on MC Length

MC Length	Testing Days
21-23	5-18
23-28	7-20
29-35	8-25
36-50	8-30

Timepoints. Two timepoints (i.e., timepoint 1 (T1) and timepoint 2 (T2)) assessed energy availability and its components and included two laboratory visits. Participants were required to fast for 12 hours prior to both visits and all visits occurred as close to waking as possible which took place between 0500-1000. Each visit marked the first day of the timepoint. T1 started in the follicular phase between D2-4 when estrogen and progesterone should be low, with estrogen starting to increase at the end of T1 while T2 started between D5-8 post ovulation when estrogen and progesterone should be high (Figure 5.2). Each visit (T1 and T2) consisted of ARSS, RHR, blood pressure, resting metabolic rate (RMR) and dual energy x-ray absorptiometry (DXA) assessments. Additionally, serum was collected at the T2 visit. At home ovulation tests determined the start of T2 (i.e., D5-8 post ovulation). If ovulation did not occur based on our criterion time frame (Table 5.1), the cycle was considered anovulatory and T2 measurements occurred no later than two days after the final ovulation test. Energy intake was

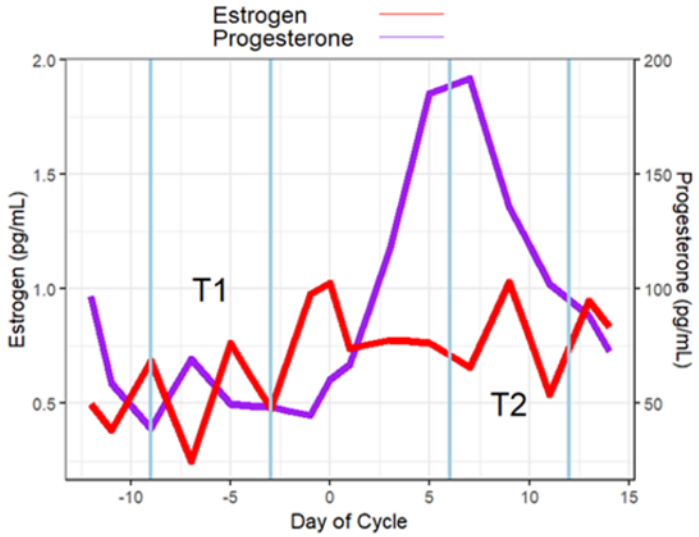
recorded for 7 days during each timepoint, starting the day of the laboratory visit unless an anovulatory cycle or late ovulation occurred, then energy intake recording started prior to the visit. Energy availability was calculated for each day energy intake was recorded in T1 and T2.

Figure 5.1. Research Study Design



Note. This protocol is based off a 28-day cycle with ovulation occurring at D14. Collection days were adjusted per menstrual cycle and ovulation. Timepoint 1 (T1) started between D2-D4 in the follicular phase; Timepoint 2 (T2) started between +D5-8. D1: day of onset of menses; +: post ovulation.

Figure 5.2. An Example of the Salivary Estrogen and Progesterone Profiles Across a Single Menstrual Cycle for One Participant



Note. Progesterone AUC: T1, 325.97; T2, 853.44. Progesterone mean: T1, 52.82; T2, 142.19. Estrogen AUC: T1, 3.19; T2, 4.56. Estrogen mean: T1, 0.54; T2, 0.76. Cycle days are centered on day of ovulation (day=0). Blue lines indicate start and end of each timepoint. T1, Timepoint 1, Day 4-10 (day -9 to -3 pre ovulation on graph); T2, Timepoint 2, Day 6-12 post ovulation. Values are presented as pg/mL.

Study Procedure

Participants. Participants were recruited from UNCG via word of mouth, emails, and flyers. This study was approved by the Institutional Review Board and all subjects provided written informed consent prior to enrollment in the study. Screening procedures consisted of a modified American College of Sports Medicine (ACSM) Exercise is Medicine Health History Questionnaire and questions regarding menstrual cycle, exercise, and medical health history. Inclusion criteria were as follows: (1) have a menstrual cycle between 21-50 days, (2) age 18-35 years, (3) a minimum of 2.5 hours of exercise per week (approximately 30-45 min per day or more) and must be a habitual exerciser for at least 6 months, (4) have no history of metabolic or cardiovascular diseases, eating disorders, or polycystic ovary syndrome (PCOS), (5) not take any hormonal contraceptives for 3 months prior to the start of the study or take any medications that would alter the metabolic or reproductive hormones (e.g. anxiety, depression, stimulants), (6) must not currently be using tobacco products (e.g. smoking, vaping), (7) not be actively dieting to lose weight, (8) are not or do not plan to become pregnant during the duration of the study and (9) have no internal metal (e.g. hip replacement, fixation of the spine).

Menstrual Cycle Characteristics

A menstrual cycle was defined as the first day of menses (Day 1) until the day prior to onset of the next menses. For this study, the follicular phase was defined as D1 through the day of the LH surge (i.e., indicating ovulation) regardless of length and T1 was measured during the follicular phase. The luteal phase is the day after the LH surge until the day before the onset of menses regardless of length. Luteal phase days are referred to as days post ovulation but if ovulation did not occur, phases could not be determined. T2 was expected to be measured in the luteal phase based off the at home ovulation tests but ovulation could not be confirmed in all participants.

Participants verified having consistent menstrual cycles every 21-50 days or were asked to track one menstrual cycle prior to starting the study. The menstrual cycle length 21-50 days was selected because even though a 'normal' menstrual cycle is between 21-35 days, it is also considered normal for menses to fluctuate up to a cycle length of 45 days for the first few years after menarche (Klein et al., 2019). Additionally, this allowed participants with potentially subclinical menstrual dysfunction to participate. Menses was logged into Training Peaks while menstrual cycle function and history was assessed once with the Low Energy Availability in Females Questionnaire (LEAF-Q) (Melin et al., 2014) prior to the first visit. Participants were instructed to contact the research team on the first day of menses to schedule subsequent visits.

Menstrual Cycle Classification

Ovulation testing days were adjusted based on the length of the previous self-reported menstrual cycle (Table 5.1). Ovulation testing days were selected as the highest probability of ovulation occurring within that menstrual cycle length (Soumpasis et al., 2020). Participants texted a daily picture of each ovulation test to the research team via WhatsApp and continued testing daily until the research team confirmed a positive ovulation or the number of testing days had been reached. If the participant failed to text a picture for two days, the research team contacted the participant. At home ovulation testing has proven to be accurate but ovulation was also confirmed by serum progesterone post study. Failure to receive a positive ovulation test and either a serum progesterone > 3.0ng/mL (American Society for Reproductive Medicine and the Society for Reproductive Endocrinology and Infertility, 2021) or an increase of salivary progesterone that was 2 SD above the mean of progesterone post ovulation (Ellison, 1988), resulted in the cycle being classified as anovulatory. A luteal phase deficit (LPD) was defined by < 10 days post ovulation to the start of the next menses (De Souza et al., 1998b).

Anthropometrics

Nude body mass was measured to the nearest 0.1 kg on a digital scale (WB-800S Plus; Tanita Corporation, Tokyo, Japan) at each visit while height was measured by a wall mounted stadiometer (Model216; Seca, Chio, CA) to the nearest 0.5 cm at the beginning of the study. Body mass was also calculated via the DXA analyses. Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2).

Body Composition (DXA)

Dual-energy X-ray absorptiometry (DXA) (Lunar Prodigy Advance, GE Healthcare, Madison, Wisconsin) was utilized to determine whole-body composition by a certified operator. Participants were fasted for a minimum of 2 hours prior to the visit and a pregnancy test was administered prior to the scan to confirm that the participant was not pregnant. Each participant wore a gown and removed all metal before the scan, which was performed twice in one menstrual cycle (C2) (i.e., T1, T2) to determine if body composition changed across the menstrual cycle. The DXA scanner has a <1% coefficient of variation for body composition measurements. Body composition was calculated using enCORE 2011 software (version 13.60) by one researcher.

Energy Availability

Energy availability describes the amount of energy available for metabolic functions after exercise energy expenditure (EEE) is subtracted from energy intake, and is expressed relative to fat-free mass (FFM) (Loucks et al., 1998).

Energy availability = [Energy intake (kcal) – EEE (kcal)]/ FFM (kg)

Although there is widely accepted threshold for energy availability, the recommendation for adequate energy availability is ≥ 45 kcal/kg FFM/day whereas ≤ 30 kcal/kg FFM/day and has been recommended as the threshold for low energy availability.

Energy availability was calculated for 7 consecutive days during T1 and T2 during C2. The average FFM from T1 and T2 from the DXA analysis was used. Energy intake and exercise energy expenditure collection procedures are listed below.

Energy Intake

Energy intake was assessed for seven days at two timepoints (i.e., T1, T2) across one menstrual cycle. Due to subject burden and to increase compliance, only two timepoints were assessed and seven days was chosen to best evaluate the association of energy intake and exercise. Energy intake was self-logged in MyFitnessPal and began the day of each follicular and luteal phase laboratory visit, whenever possible. If the participant was anovulatory or had a late ovulation, energy intake was recorded prior to the T2 visit to allow the full seven days to be logged. Participants received detailed instructions from the researcher on how to log all food and beverages as well as a handout with portion sizes. The same researcher also confirmed MyFitnessPal entries at the next visit to ensure accuracy. MyFitnessPal is a validated smartphone app with reliable dietary analysis (Evenepoel, Clevers, Deroover, Loo, et al., 2020; Evenepoel, Clevers, Deroover, Matthys, et al., 2020; Teixeira et al., 2018). Total energy intake and macronutrient composition were calculated.

Physical Activity

Wrist-worn actigraphy (ActiGraph GT9X Link) assessed total daily activity for 7 days, starting the day of the T1 visit. Participants were instructed to continuously wear the accelerometer on their nondominant wrist, except for activities that involved water (i.e., swimming, showering). Accelerometers were programmed and downloaded using Actigraph software. The raw data analyses were performed with R- package GGIR (Hees et al., 2013) that was expressed in gravitational equivalent units called milli-gravity (*mg*, where 1000*mg* = 1*g* = 9.81 m/s^2). To be included in the analyses, participants needed four days that included a

minimum of 16 hours each day. Currently, there are no criterion for categorizing total physical activity (i.e., low, high) when calculating the physical activity with raw data. Therefore, the raw data is used to assess total physical activity, with higher values indicating higher physical activity.

Exercise

Participants were given a Polar H10 heart rate monitor (Polar H10 Heart Rate Sensor; Polar Electro Inc., Bethpage, NY) to wear during all purposeful exercise greater than 10 minutes in duration. Purposeful exercise can include activities such as jogging, strength training, etc. but not daily living activities such as house cleaning. The heart rate monitor was linked to a recording device of the participants choice (e.g., Polar Beat, Garmin, Apple) that automatically uploaded data to the Training Peaks (TP) app. Ratings of perceived exertion (RPE) was recorded using a built-in function of TP that utilized a modified sliding scale of 0-10 for each exercise session.

Calculation of exercise energy expenditure used training heart rate divided into 7 heart rate zones equally distributed between resting heart rate (RHR) and maximum heart rate. For each heart rate zone, the metabolic equivalent (MET) was calculated using the heart rate index method ($6*(HR_{absolute}/HR_{rhr})-5$). This method has been validated and shown to be an accurate measure of VO_2 at different intensities (KANG et al., 2020; Wicks et al., 2011; Wicks & Oldridge, 2016). A MET is approximately equal to a resting value of 3.5 ml/kg/min but this varies by participant and females typically have lower values (Byrne et al., 2005). Therefore, corrected METs were calculated using the measured resting VO_2 (MET * 3.5ml/kg/min / resting VO_2 ml/kg/min). Kilocalories from exercise were quantified using the corrected METs multiplied by exercise duration (min) and weight (kg). MET values contain resting values, therefore measured

resting energy expenditure (kilocalories/min) were subtracted from the exercise kilocalories to obtain the correct exercise energy expenditure (Reed et al., 2015).

If heart rate was not recorded, the participant logged a description of the activity, duration, intensity, and RPE in training peaks which was used to determine the appropriate METs with the compendiums of physical activities (Ainsworth et al., 2011). Corrected METs were calculated prior to calculating kilocalories as described above by the same researcher. TP software calculated training stress score (TSS), an estimate of the training load based off intensity and duration that is commonly used to determine how much recovery may be needed after an exercise session. TSS is calculated off exercise heart rate. (*TrainingPeaks*, n.d.).

Acute Recovery and Stress Scale (ARSS)

The Acute Recovery and Stress Scale (ARSS) assessed the current recovery-stress state of an athlete at an emotional, mental, and physical level (Appendix C). Subscales of performance capability, mental performance capability, emotional balance assessed recovery while stress subscales consisted of muscular stress, lack of activation, negative emotional state, and overall stress and descriptives of each subscale are listed in Table 5.2. The subscales in both stress and recovery were added together to assess total stress and total recovery (Kellmann & Kolling, 2019). The stress-recovery state was calculated by subtracting recovery from stress, with 0 indicating a balanced relationship, a negative number indicating higher stress than recovery, and a positive number indicating a higher recovery than stress state. The ARSS survey was administered as an electronic survey via Qualtrics using a Likert scale ranging from zero (does not apply at all) to six (fully applies). Each subscale is comprised of four questions with a maximum score of 24 for each subscale. ARSS has a Cronbach's α that ranges between .77-.88 (Kellmann & Kolling, 2019). The ARSS was taken on the first and last day of T1 and T2 in one menstrual cycle. The survey was administered during the laboratory visit at the beginning

of each timepoint and the participant was sent a link to the survey via WhatsApp the morning of the last day of T1 and T2.

Table 5.2. Overview of ARSS Subscales Adjectives

Subscale	Descriptive
Recovery	
Physical Performance Capability	Strong, energetic, physically capable, full of power
Mental Performance Capability	Attentive, receptive, concentrated, mentally alert
Emotional Balance	In a good mood, have everything under control, stable, pleased
Overall Recovery	Recovered, rested, muscle relaxation, physically relaxed
Stress	
Muscular Stress	Muscle exhaustion, muscle fatigue, muscle stiffness, muscle soreness
Lack of Activation (Motivation)	Unmotivated, sluggish, unenthusiastic, lacking energy
Negative Emotional State	Feeling down, short-tempered, stressed, annoyed
Overall Stress	Tired, worn-out, overloaded, physically exhausted

Hormones

Blood Collection and Preparation. Participants reported to the UNCG Exercise Endocrinology Laboratory between the hours of 0500-1000 for one visit during the following ranges: D2-4, D9-11, and two consecutive days between D5-8 post ovulation then again between D5-8 post ovulation (i.e., T2) in the other menstrual cycle for a total of 5 blood samples. Participants were instructed to be fasted for 8 hours prior to arrival. Approximately 10mL of blood was collected in a serum blood collection tube. Blood samples were allowed to clot for 20 min at room temperature then centrifuged at 3000 rpm for 15 min at 4°C. The serum samples were aliquoted into multiple 2 mL polyethylene storage tubes and frozen at -80°C.

Saliva Collection and Preparation. Saliva collection was chosen to measure the daily hormonal profile for one menstrual cycle to reduce invasiveness and increase participant compliance. Measurements of E2 and progesterone in saliva have a strong correlation with serum concentrations in premenopausal women and additionally are stable at room temperature for a few days and at -20°C long-term (Bellem et al., 2011). Serum was collected to confirm the correlation between saliva and serum.

Saliva was collected every day via passive drool using polyethylene storage tubes with straws supplied to the participant by the investigator. Participants were instructed to collect their saliva immediately after waking and to refrain from brushing teeth, eating, or drinking until the sample is collected. Participants stored saliva samples in a home freezer (-20°C) until they were returned to the research team at every visit, then the samples were stored at -80°C. Saliva samples were thawed at room temperature and then centrifuged at 13,000 rpm for 15 minutes prior to assay.

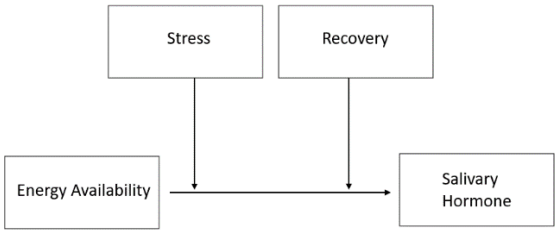
Serum and Saliva Measurements. Estrogen (17-β estradiol) and progesterone were quantified in serum and saliva samples by enzyme immunoassay (Immuno-Biological Laboratories, Minneapolis, MN (serum); Salimetrics, Carlsbad, CA (saliva)). All hormone determinations were assayed in duplicate with all samples from a given participant on the same assay whenever possible. The sensitivity of the serum and saliva E2 assays are < 1.399 pg/mL and 0.1 pg/mL and the progesterone assays have a sensitivity of 0.045 ng/mL and 5 pg/mL, respectively. Samples were reanalyzed if a coefficient of variation was > 25% for saliva and > 20% for serum. The intra-assay coefficients of variation for low and high controls were 9.6% and 3.3% (saliva progesterone), 3.1% and 5.9% (saliva estrogen), and 19.2% and 5.7% (serum estrogen) respectively. Inter-assay coefficients of variation for low and high controls were 19.6% and 34.2% (saliva progesterone), 13.5% and 16.6% (saliva estrogen), and 22.9% and 22.5% (serum estrogen) respectively. Serum hormones were used to verify ovulation and salivary hormones gave a hormonal profile for each menstrual cycle.

Statistical Analysis

Statistical analyses were conducted using SPSS Statistics 24 (IBM, Armonk, NY, USA) and R Statistical Software (v2022.12.0; R Core Team 2021). Data was summarized as mean ± SD.

A repeated measures analysis of variance (ANOVA) was performed to examine differences between the ARSS stress and recovery scores at four timepoints (i.e., beginning and end of T1 and T2). A Bonferroni post hoc testing was used to reveal where significant differences occurred. To determine if the effects of energy availability on hormones would be affected by stress and recovery, the PROCESS moderation (model 2) (Figure 5.3) was used with a boot strapping technique and the Johnson-Neyman technique for continuous moderators (A. F. Hayes, 2012). Two timepoints were assessed in one menstrual cycle that consisted of seven days each time. T1 started between D2-4 while T2 started between D5-8 post ovulation. Estrogen and progesterone were measured with area under the curve at each timepoint while the average of energy availability was taken at each timepoint. Stress and recovery were separate moderators in the same model, that was assessed with ARSS total stress and total recovery scores from the survey given at the beginning of each timepoint. A separate model was used for each hormone and each timepoint, for a total of 4 analyses.

Figure 5.3. PROCESS Model 2 with Stress and Recovery Scores as Moderators



The above analysis was repeated with the PROCESS moderation (model 1) (Figure 5.4 and 5.5) that assessed the effect of stress as a single moderator on the energy availability and hormone relationship. This time the stress moderator was TSS (Figure 5.4), a value of the stress from the exercise training load. A third analysis was completed with the PROCESS moderation (model 1) (Figure 5.5) stress-recovery state as the stress moderator. The stress-

recovery state was calculated by subtracting recovery from the ARSS stress, with 0 indicating a balanced relationship and a negative number indicating higher stress than recovery.

Figure 5.4. PROCESS Model 1 with TSS as the Moderator

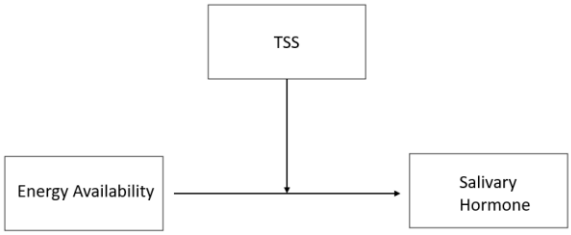
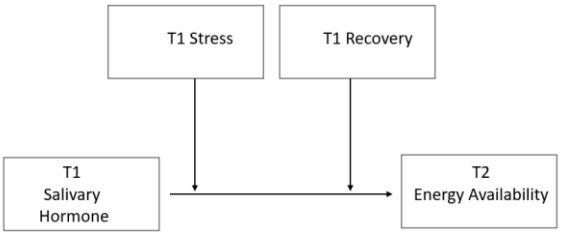


Figure 5.5. PROCESS Model 1 with the Stress-Recovery Score as the Moderator

A secondary analysis further investigated the hormone and energy availability relationship on a relationship that was discovered in Chapter 4. T1 hormones had a negative relationship with T2 energy availability while controlling for T1 energy availability. Therefore, stress and recovery moderators were added to this relationship to evaluate how stress and recovery influenced this relationship (Figure 5.6). Total stress and recovery scores were still used in Model 2. This relationship was also examined using PROCESS moderation model 1, with TSS, stress-recovery state, and physical activity analyzed in separate models. Physical activity was only included in this model as a moderator.

Figure 5.6. PROCESS Model 2 with Stress and Recovery Scores as Moderators



Results

Demographic and Reproductive Characteristics

The demographic and reproductive characteristics are shown in Table 5.3. Twenty-one participants completed the study but due to one participant having low compliance with collecting saliva, only 20 participants are used for analyses. Participants identified themselves as Caucasian (43%), Hispanic (19%), African-American (33%), and an unlisted race (5%). Weight, BMI, body fat %, or fat free mass did not change across the cycle ($p > .05$) therefore the average was taken and presented in Table 5.3.

Table 5.3. Demographic and Reproductive Characteristics

	n=21
Age (years)	21 ± 3
Height (cm)	163.2 ± 4.5
Weight (kg)	63.22 ± 11.4
BMI (kg/m^2)	23.8 ± 4.0
Body fat (%)	30.0 ± 8.9
Fat free mass (kg)	43.4 ± 5.8
Physical activity (*mg*/d)	40.8 ± 14.7
Age of menarche (years)	11 ± 1
Gynecological age (years)	9 ± 3
Menstrual cycle length (d)	29.6 ± 3.4

Values are mean ± SD.

All participants were nulliparous and underwent menarche prior to 15 years of age. Three out of 21 cycles were categorized as anovulatory (14%) while 18 menstrual cycles were confirmed as ovulatory (86%). No cycles were classified as LPD within this menstrual cycle.

Physical activity was expressed in gravitational equivalent units called milli-gravity (*mg*, where 1000*mg* = 1*g* = 9.81 m/s^2) and is a different measurement than the traditional counts and therefore cannot be directly compared. Research is lacking using this method with physically active females with most of it focusing on children and older adults, but for reference, research that used the same age group of females (average age 22 years) of sedentary females averaged 32.4 *mg*/d of physical activity (Acosta et al., 2019).

Energy Availability and Hormone Characteristics

Energy availability measures were obtained during a single menstrual cycle only and at two timepoints throughout that cycle and are summarized in Table 5.4. The first measure was timepoint 1 (T1) that started between D2-4 after the start of the menstrual cycle, when estrogen and progesterone are low (early follicular phase). The second timepoint (T2) started between D5-8 post ovulation. T2 should be capturing the luteal estrogen and progesterone peak. However, the start of T2 was based off at home ovulation tests and serum hormones could not confirm all participants met criterion measures. Thus, although T1 is definitively representative of the follicular phase, T2 did not always definitively represent the luteal phase and T1 and T2 will be used instead of follicular phase and luteal phase.

No changes between timepoint were significant for energy availability, energy intake, or exercise (p>.05). Progesterone AUC, progesterone range, and estrogen AUC was higher as expected in T2 compared to T1 (p=.001, p=.027. p=.003). Most participants (71%; 15/21) were in a reduced energy availability state, with 19% (4/21) classified as low energy availability (< 30 kcal/kg FFM) while only 10% (2/21) were above the recommended 45 kcal/kg FFM. Only one participant with menstrual dysfunction was classified as low energy availability.

Participants completed ~3 hours of exercise per week, slightly exceeding the 2.5 hours ACSM recommendations for exercise. Strength training was the primary mode of physical

activity for most of the participants (81%). Other activities included running (57%), yoga (29%), indoor cycling (31%), soccer (19%), Zumba (14%), and dance (10%). Compared to previous research that investigated active eumenorrheic females with similar energy availability (~36 kcal/kg FFM/d) (Reed et al., 2011), our participants expended less energy during exercise (156 kcal, compared to 296 kcal). TSS is a training load index based off heart rate and duration and is correlated with RPE. Although there is no literature investigating TSS in physically active females, female pro cyclists averaged 224 TSS in a 3-hour road race (Sanders et al., 2019). Recreational male cyclists that trained at least 10 hours per week averaged 766 TSS per week (Woods et al., 2018) which is significantly higher than our population, who averaged 174 TSS with 3 hours of exercise per week. Higher RPE was associated with higher TSS in T1 ($r = .42$, $p = .002$) and T2 ($r = .31$, $p = .042$) as expected.

Table 5.4. Energy Availability and Salivary Hormone Characteristics at T1 and T2 Across One Menstrual Cycle

	T1	T2	p	Mean
EA (kcal/kg/FFM/d)	35.8 ± 11.5	35.8 ± 7.5	.993	35.8 ± 8.8
Energy Intake (kcal/d)	1682.6 ± 390.3	1667.6 ± 179.8	.855	1675.1 ± 240.1
Carbohydrate (% kcal/d)	45.0 ± 6.2	44.8 ± 7.2	.849	44.9 ± 6.2
Fat (% kcal/d)	38.5 ± 6.6	36.3 ± 7.5	.281	37.4 ± 5.4
Protein (% kcal/d)	17.4 ± 4.3	16.7 ± 5.7	.430	17.0 ± 4.7
Protein (g/kg/d)	1.2 ± 0.5	1.1 ± .37	.860	1.2 ± 0.4
Exercise duration (hour/week)	3.2 ± 1.8	2.5 ± 1.9	.190	2.9 ± 1.4
EEE (kcal/d)	168.9 ± 142.1	143 ± 152.0	.255	156.0 ± 138.3
Total TSS	180 ± 122.5	167.4 ± 137.7	.693	173.7 ± 108.7
Progesterone AUC	1197.4 ± 932.0	1765.17 ± 1033.9	**.001**	
Progesterone range	154.3 ± 109.4	226.8 ± 136.4	**.027**	
Progesterone minimum	128.4 ± 122.8	180.9 ± 137.1	.094	
Progesterone maximum	282.7 ± 174.1	407.7 ± 223.0	**.001**	
Estrogen AUC	8.3 ± 4.4	10.5 ± 4.2	**.003**	
Estrogen range	.8 ± .6	1.0 ± .7	.176	
Estrogen minimum	1.0 ± .6	1.2 ± .5	**.017**	
Estrogen maximum	1.8 ± .8	2.2 ± .9	**.004**	

Values are mean ± SD. d, day; EA, energy availability; EEE, exercise energy expenditure; TSS, exercise training stress score. *all hormones are presented as pg/mL, n=20. All other values are n=21.

Acute Recovery and Stress Scale

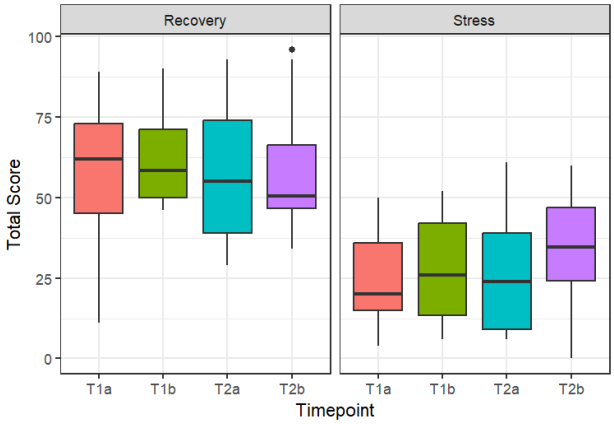
The ARSS survey was administered at 4 timepoints across one menstrual cycle. Each survey was administered at the start and end of T1 and T2. The results are presented in Table 5.5. A repeated measures ANOVA revealed that the stress subscale, negative emotional state, was lower in T1a and T2a compared to T2b (p = .000). Total stress scores were significant (p = .032) across the cycle but a post-hoc analysis revealed no difference between timepoints. Total recovery remained constant across the cycle (p > .05) (Figure 5.7). The other stress and recovery subscales remained constant across the cycle. The stress-recovery state indicated participants were consistently in a low stress, high recovery state for one menstrual cycle.

Table 5.5. ARSS Results over 4 Timepoints Across a Menstrual Cycle

Subscale	T1 (start)	T1 (end)	T2 (start)	T2 (end)	p
Recovery					
Physical Performance Capability	13.5 ± 6.2	14.9 ± 4.3	13.6 ± 6.3	12.9 ± 6.1	.402
Mental Performance Capability	15.9 ± 5.4	16.7 ± 3.5	15.6 ± 5.6	15.2 ± 5.1	.606
Emotional Balance	14.5 ± 5.3	16.2 ± 3.9	15.8 ± 5.6	14.8 ± 4.7	.552
Overall Recovery	13.8 ± 5.4	14.0 ± 4.3	14.1 ± 5.1	13.7 ± 4.7	.992
Total Recovery	58.7 ± 20.8	61.8 ± 12.4	59.1 ± 20.9	56.6 ± 18.7	.637
Stress					
Muscular Stress	4.4 ± 3.2	5.5 ± 4.8	4.6 ± 4.4	7.4 ± 5.6	.073
Lack of Activation (Motivation)	7.3 ± 5.0	6.7 ± 4.5	6.4 ± 5.8	8.3 ± 5.2	.460
Negative Emotional State	4.5 ± 3.5*	5.7 ± 4.8	4.3 ± 4.2*	7.8 ± 4.6	**.000**
Overall Stress	9.0 ± 6.1	9.2 ± 4.6	8.9 ± 5.9	10.4 ± 5.5	.573
Total Stress	25.1 ± 15.0	27.1 ± 15.3	24.3 ± 16.1	33.9 ± 17.0	**.032**
Stress-Recovery State	33.6 ± 33.5	34.7 ± 24.0	32.7 ± 32.1	22.7 ± 32.2	.228

Values are mean ± SD. Stress-recovery state, - indicates higher stress than recovery. * Timepoints significantly different from T2(end), p<.05. n=19. See Table 5.2 for descriptions of the subscales. A total score of 24 was possible in each subscale and a possible score of 96 for total recovery and stress. The higher the value on the scale, the higher is the current recovery or stress state in that area with the exception of the stress-recovery state where a positive value indicates a higher recovery rate while a negative value indicates a higher stress state.

Figure 5.7. Box and Whisker Plot Depicting Total Recovery and Stress Scores Across One Menstrual Cycle



Note. a, start of timepoint; b, end of timepoint.

Influence of Stress and Recovery on Energy Availability, Estrogen, and Progesterone

A Pearson correlation was used to determine if there was any association between energy availability, progesterone, and estrogen with the stress and recovery score (Table 5.6 and 5.7). Total stress scores were inversely associated with estrogen (r = -.521, p=.018) and progesterone AUC (r = -.478, p=.033) in T1 but not T2, indicating that at the beginning of the cycle, when total stress was high, estrogen and progesterone AUC was low. Yet total recovery scores were not correlated with either hormone. TSS (i.e., exercise training stress) was not correlated with any ARSS subscales. No other significant correlations were found (p>.05), suggesting that there are not linear relationships between energy availability, estrogen, and progesterone with stress and recovery scores within a timepoint, except for total stress with progesterone and estrogen in T1.

Table 5.6. Correlations of Energy Availability, Salivary Hormones, and Stress and Recovery Scores in T1

	1	2	3	4	5	6	7
1. EA	1.00						
2. Progesterone AUC	0.21	1.00					
3. Estrogen AUC	0.03	**.74****	1.00				
4. Total Recovery Score	-0.19	0.20	0.30	1.00			
5. Total Stress Score	-0.01	**-.48***	**-.52***	**-.71****	1.00		
6. Stress-recovery state	-0.11	0.34	0.42	**.95****	**-.90****	1.00	
7. TSS	-0.28	0.14	0.10	0.02	0.00	0.01	1.00
N	21	20	20	21	21	21	21

*, p<.05; **, p<.01; EA, energy availability; AUC, area under the curve; TSS, exercise training stress score

Table 5.7. Correlations of Energy Availability, Salivary Hormones, and Stress and Recovery Scores in T2

	1	2	3	4	5	6	7
1. EA	1.00						
2. Progesterone AUC	-0.13	1.00					
3. Estrogen AUC	-0.25	**.49***	1.00				
4. Total Recovery Score	-0.10	0.13	-0.02	1.00			
5. Total Stress Score	0.19	-0.17	-0.16	**-.44***	1.00		
6. Stress-recovery state	-0.16	0.17	0.07	**.87****	**-.82****	1.00	
7. TSS	-0.32	-0.17	-0.24	-0.01	-0.13	0.06	1.00
N	21	20	20	21	21	21	21

*, p<.05; **, p<.01; EA, energy availability; AUC, area under the curve; TSS, exercise training stress score

A moderation model was used to assess if stress and recovery affected the relationship between energy availability and estrogen and progesterone. A separate model was used for each hormone (AUC) and timepoint. The ARSS total stress and recovery scores at the beginning of each timepoint were used as the moderators. No models were significantly different from zero and no significant interactions with stress or recovery were noted at T1 or T2 with either hormone ($p > .05$). This indicates that within a timepoint, there is not a direct relationship between energy availability and hormones and stress and recovery do not moderate this relationship. No direct relationships or interactions were significantly different from zero ($p > .05$) when Model 1 was used with either TSS or the stress-recovery state as moderators.

A previous analyses (Chapter 4) revealed there was a negative relationship with T1 hormones with T2 energy availability, while controlling for T1 energy availability. Therefore, this

analysis was rerun using that model to determine if stress and recovery changed the relationship. Total stress and recovery scores were utilized in a Process 2 model then the stress-recovery scores, TSS, and physical activity were used in a Process 1 model. No significant interactions emerged in any model ($p > .05$), indicating that TSS, stress and recovery, and physical activity do not moderate the relationship between hormones and energy availability.

Discussion

This study explored the relationship of energy availability with estrogen and progesterone and the influence of stress and recovery on that relationship across the menstrual cycle. Total stress and a negative emotional state were highest towards the end of the menstrual cycle while recovery remained constant. The stress-recovery state indicated a steady higher recovery and lower stress state across the cycle. Energy availability did not change across the cycle while estrogen and progesterone were increased post ovulation. Total stress was negatively associated with estrogen and progesterone in T1 was the only relationship that emerged within a timepoint when stress, recovery, energy availability, and hormones were assessed. Stress and recovery did not moderate the relationship with energy availability within or across timepoints.

We hypothesized that energy availability would predict estrogen and progesterone hormone concentrations and this relationship would be stronger with increased recovery and decreased stress scores and this hypothesis is rejected. Energy availability did not predict either hormone at T1 or T2 and the relationship was not changed by recovery and stress scores. When the association of T1 hormones were assessed with T2 energy availability while adjusting for T1 energy availability, the previous direct relationships emerged but stress and recovery did not moderate the relationship with either hormone. There were no correlations between stress,

recovery, or the stress-recovery state with energy availability and the AUC of estrogen or progesterone within a timepoint except for T1 total stress with T1 hormones, which explains the lack of influence on the energy availability and hormone relationship. Stress was expected to affect the relationship with energy availability and hormones due to previous literature indicating stress can affect hormones across the menstrual cycle (Zavala et al., 2020) and under-recovered states have been associated with menstrual dysfunction (Schaal et al., 2021). Yet, exactly how the relationship between energy availability and hormones are influenced by stress and recovery was unknown.

A relationship may exist between energy availability, hormones, and stress and recovery states but the participants in this study may not have performed a high enough training load or had a low enough reduced energy availability state to be affected by stress and recovery. Participants in this study were primarily in a reduced energy availability state, but a relationship may become 'visible' when females in a low energy availability state are examined. Also, the ARSS was designed to assess stress and recovery in athletes but has primarily been validated in elite athletes. Our participants exercised on average between 2-3 hours per week and the ARSS might not have been sensitive enough to detect changes in this population due to our population of physically active females occurring less training stress than elite athletes. In addition, the training load (TSS) was not associated with any of the ARSS scores. This be partially due to the average TSS for the week was used for analysis while investigating the TSS and ARSS survey on the same day might be more relevant. However, participants exercised at their discretion and did not always exercise the same day the ARSS was administered. When the ARSS was administered at the morning of the T1 visit, no exercise had occurred prior to the visit. The training load may not have been large enough to affect stress and recovery scores, with the participants staying in a recovered state for the duration of the study. Furthermore,

participants in this study exercised an average of 3 hours per week and may not have had a high enough intensity or volume to produce a significant response for stress and recovery. Without any previous research to compare to, further research with low energy availability is needed in females that are considered physically active. Collecting data on this specific group (LEA physically active) may require utilization of females that are training for competitions, or specifically doing endurance activities, where high volumes of training are required even for relatively recreational levels of athletes. Previous research investigated 35 recreational athletes (18 male/ 17 female) with an average age of 48 that exercised at least three times a week. Participants were given the ARSS 10 weeks apart in an observation study and no changes in any stress or recovery subscales were noted (McGuigan et al., 2022). However, the participants subscale scores appear to be higher than our population. For example, the pre scores for overall stress and overall recovery were 16.6 and 13.5 respectively, while our participants had total scores of 13.8 and 9.0 at the start of the study. A maximum score of 24 is allowed for each subscale. Age or exercise hours per week could be a factor for the increased scores in the previous study. Participants were twice the age of our participants and exercised an average of six hours per week and both age and exercise volume could influence stress and recovery—individuals in this higher age range were likely to have significant 'life stress' (i.e., demanding careers, children, household duties), in addition to exercise stress, which could contribute to the higher stress scores. In addition, these older individuals were likely to have years of experience balancing recovery with exercise and likely had a more 'seasoned' approach to recovery, accounting for their higher recovery scores as well. The ARSS was given to male and female athletes that ranged from recreational to international level athletes one time (Kölling et al., 2020). The mean score per subscales were used, not the total score as previously reported here, where a maximum score of six is possible. Mean scores for overall stress (2.4 vs 2.2) and

recovery (3.5 vs 3.5) were similar to our results. Albeit it is difficult to directly compare due to only 9% of that population being classified as recreational athletes, but it shows that our stress and recovery scores are comparable to other athletes.

Even though no direct relationships were discovered, the participants negative emotional state (i.e., feeling down, stressed, annoyed, short-tempered) changed across the cycle while all other stress and recovery subscales remained constant. Negative emotional state scores were highest towards the end of the menstrual cycle, which is the part of the cycle that is associated with premenstrual syndrome (PMS). PMS is characterized by emotional, physical, and behavioral symptoms that occur during the luteal phase and stops within a few days of the onset of menses (Yesildere Saglam & Orsal, 2020). When assessing eumenorrheic and oligomenorrheic women, a previous study showed that both groups had an increased negative mood state in the later luteal phase compared to midluteal that was associated with PMS (Cockerill et al., 1992). The start of T2 would be considered midluteal when the ARSS was first administered, then seven days later it was administered again which should have been late luteal phase. Identifying PMS was outside of the scope of this study but the change in the participants negative emotional state could be associated with PMS and warrants further investigation.

No other stress and recovery subscales changed across the cycle and this corresponds with a recent meta-analysis that indicated stress, muscle soreness, and fatigue did not change from the early follicular to late luteal phase (Paludo et al., 2022). However, only 14 studies were included in the meta-analysis with a variety of outcome variables (e.g., motivation to train, mood, RPE) and five studies measured RPE as the only outcome. Furthermore, very few studies confirmed menstrual phases with hormones (e.g., at home ovulation tests) and most used the calendar counting method (e.g., luteal measurement at day 21) which does not

account for variation in menstrual cycles and could potentially be assessing participants in different phases of the menstrual cycle.

Limitations and Future Directions

Our measurements were assessed over one menstrual cycle, but it is recommended that measurements should be repeated in an additional menstrual cycle. Due to the variation between cycles, it is recommended to repeat the outcome measures in a second menstrual cycle (Elliott-Sale et al., 2021) and a second cycle would confirm hormones, energy availability, and stress and recovery values. A second menstrual cycle could potentially help determine if the change in negative emotional state was due to the menstrual cycle or external stressors. Our population consisted of undergraduate and graduate students that were started on a rolling basis; therefore, participants began the study at different timepoints between the beginning of the semester and mid-semester. Students typically experience higher academic demands at the end of the semester (Pope & Harvey-Berino, 2013) and having some participants finish at the middle of the semester versus the end of the semester when demands are higher could have a potential effect on the results.

Assessing stress and recovery at only 4 timepoints may not be enough to effectively capture the changes across the cycle. Daily ARSS surveys taken each day energy availability was measured would allow for a more comprehensive view of the relationship and would allow daily or every other day measures to be assessed between energy availability, hormones, and stress and recovery. In active individuals, stress and recovery as well as energy availability fluctuates daily based off training load and should be investigated with the daily variability of hormones. In a study investigating stress and recovery in swimmers over 17 weeks, large differences were found between swimmers despite that they followed the same training protocol. Therefore the researcher recommended that individual evaluations as well as repeated

measures should be used when assessing stress and recovery (Collette et al., 2018). This also holds true with investigating the effect of physical activity on hormones and energy availability. Physical activity and exercise are highly variable. In our study, exercise activities alone were diverse with multiple participants participating in more than one type of exercise. Furthermore, duration and intensity are not always the same for every exercise session and all these factors can influence energy availability. Due to high daily variation of physical activity, exercise and energy availability, a daily measurement of stress and recovery should be assessed.

Total stress scores were correlated with estrogen and progesterone in T1 but not T2. A potential reason for this could be due to the variation in hormones in T2, where progesterone and estrogen is higher than T1. Due to the large variation in menstrual cycles, individual analyses should be conducted to further identify if an association exists with total stress and hormones post ovulation.

Despite the limitations listed above, this study investigated stress, recovery, energy availability, estrogen, and progesterone across the menstrual cycle. Most of the current research focuses on cross sectional data while this longitudinal study investigated two seven-day timepoints across the cycle. Each participant recorded 14 days of energy intake and exercise (seven days at each timepoint) that allowed energy availability to be assessed across the cycle. Exercise is not always consistent in intensity, duration, and number of sessions and seven days allowed that variation to be captured, therefore giving an accurate average of energy availability. Furthermore, stress and recovery were assessed at four timepoints across the cycle that gave a comprehensive view of the stress-recovery state across the cycle.

Conclusion

In summary, stress and recovery do not moderate the relationship between hormones and energy availability within a timepoint or across timepoints. A stress subscale, negative

emotional state, was significantly higher post ovulation towards the end of the cycle while recovery and other stress scales remained constant. Further studies are needed to investigate the relationships at more timepoints across the cycle to fully elucidate the variation of hormones, energy availability, and stress and recovery across the menstrual cycle.

CHAPTER VI: CONCLUDING REMARKS AND CONSIDERATIONS

General Comments

Each of these three aims were designed to provide information about the relationship between energy availability and sex steroid hormones, estrogen, and progesterone across the menstrual cycle. Aim 1 looked at the overall relationship of energy availability and hormones, Aim 2 focused on the relationship at different timepoints across the menstrual cycle, and Aim 3 investigated how stress and recovery influenced the energy availability and hormone relationship. It is clear from our findings that the menstrual cycle is a complex system with large intra- and inter-individual variation that makes defining the relationship among energy availability and hormones challenging. However, initial evidence suggests that estrogen, progesterone, and the estrogen progesterone product at the beginning of the cycle may influence energy availability post ovulation while the mechanism of this relationship is unclear.

Aim 1 looked at the overall relationship of energy availability and hormones. Participants were primarily in a reduced energy availability state. This finding alone is worrisome, indicating that physically active females are not properly balancing exercise and energy intake. While participants did not follow the typical patterns associated with low energy availability, when the six participants that experienced menstrual dysfunction were removed from the analysis, some of the expected patterns emerged. This highlights and emphasizes the importance of evaluating menstrual function when researching the menstrual cycle and separating eumenorrheic females from females with menstrual dysfunction during analysis.

Aim 2 investigated the transactional relationship of energy availability and hormones at different timepoints across the cycle. A cross-lagged analyses found that estrogen, progesterone, and the estrogen progesterone product at the beginning of the cycle had a negative relationship with energy availability post ovulation, indicating that lower hormone levels

resulted in higher energy availability post ovulation. Yet the expected relationship of energy availability influencing hormones did not occur. This novel use of cross-lagged analysis in hormone research warrants further investigation into how hormones affect energy availability and the components of energy availability (e.g., exercise energy expenditure, energy intake).

Aim 3 evaluated the influence of stress and recovery on the relationship between energy availability and hormones. Feelings of negative emotional balance (e.g., feeling down, stressed, annoyed, short-tempered) were highest at the end of the menstrual cycle. Total stress scores from the ARSS indicated that higher stress was associated with lower estrogen at the beginning of the cycle. Yet stress and recovery did not influence the energy availability relationship within a seven-day timepoint or at different timepoints across the menstrual cycle.

Rationale and Potential Impact

According to the Centers for Disease Control and Prevention (CDC), infertility effects 12% of women aged 15-44 years in the United States and has a large emotional (i.e., anxiety, depression) and financial burden for women (Greil et al., 2011; Lai et al., 2021). Subclinical menstrual dysfunction (i.e., anovulation, luteal phase defect (LPD)) account for 30% of infertility (Hamilton-Fairley, 2003)—often occurring when menstrual cycles appear normal. Furthermore, exercising females are at a higher risk for menstrual dysfunction than sedentary women. For instance, recreational female runners have a high prevalence of anovulation (79%), LPD (48%), and decreased sex steroid hormones when compared to sedentary individuals (De Souza et al., 1998a). This is a huge concern due to a large percentage of the female population participates in exercise. The CDC reported 49.3% of women over the age of 18 in the United States meet the federal aerobic physical activity (exercise) guidelines. Although exercise is associated with numerous positive health outcomes (e.g., lower cardiometabolic risk), excessively high amounts of exercise paired with inadequate energy intake are related to negative health outcomes, and

can result in low energy availability (Gibbs et al., 2013). These subtle changes in menstrual cycle function may occur with as little as 30 minutes of exercise per day in females with low body mass index (BMI) (Chavarro et al., 2007). By understanding how habitual exercise and energy availability affects healthy women with seemingly normal menstrual cycles, the risk for menstrual dysfunction and disrupted hormones will be reduced, ultimately leading to less infertility issues.

Lessons Learned

The menstrual cycle is a complex and chaotic system that requires individual analysis. When menstrual cycles are grouped together the subtle nuances within each cycle are lost and unfortunately, this is a common practice within research related to menstrual cycles. Overall hormone concentrations, ranges, and patterns vary within a person across cycles and between individuals.

While adherence was high with most aspects of this study, exercise compliance was low. During the screening process, all participants indicated performing exercise for an average of 150 minutes or more per week and exercised at least three days per week. While the average exercise was three hours per week for the study, not every participant met the expected 150 minutes per week or exercised three days per week. Participants were asked to wear a heart rate monitor during exercise so 1) exercise energy expenditure could be calculated and 2) to verify exercise was performed. Some participants struggled with wearing the heart rate monitor consistently and exercise had to be manually inputted. This caused the exercise energy expenditure to be estimated and it was unclear if the exercise was truly performed by the participant. A restriction was not placed on participants on the type or intensity required, with exercise defined as all purposeful exercise greater than 10 minutes in duration. Purposeful exercise could include activities such as jogging, strength training, etc. but not daily living

activities such as house cleaning. Some participants only performed low intensity exercise for the duration of the study and a variety of exercises (i.e., running, weight training) were performed with different intensities. To accommodate the difference in intensities within a workout, seven heart rate zones were calculated to best assess the variation in exercise energy expenditure, but this could not accurately be captured when the exercise was not recorded with a heart rate monitor. Due to the variation of training loads and exercise volume, some participants may not have had a high enough training load to elicit the responses expected with exercising females.

Future Investigations

Future studies should aim to better understand the relationship between energy availability and hormones across the menstrual cycle. Individual analysis may be required to fully understand these relationships and longitudinal studies should be performed to capture the variance of hormones across the cycle. Studies should be performed with proper methodology for menstrual cycles that assess hormones to confirm menstrual function at numerous time points across several cycles. Although at home ovulation tests are a validated method to determine ovulation, a positive ovulation test did not always appear to correctly define the luteal phase. Furthermore, a positive ovulation tests only indicates the LH surge and does not guarantee that ovulation occurred. Some participants had a positive ovulation test but did not have a progesterone peak within the 5-8 days post ovulation as expected. It is unclear if this is due to an inaccurate test, a failed ovulation, or the hormonal variation of the individual. Thus, further investigation is needed to understand the patterns of hormones post ovulation in exercising females and to determine if one potential infertility-based issue in exercising females is a disconnect between the LH surge (captured on the ovulation test) and an actual ovulatory event.

Future studies should also include daily measures to identify if a time-lagged response exists between hormones and energy availability. Our data indicates the potential of a time-lagged response between hormones at the beginning of the cycle and energy availability post ovulation, but a more in-depth investigation is needed at a daily level. In addition, the progesterone to estrogen ratio should be examined more closely.

Other biomarkers, such as TT_3 and iron, should be investigated in physically active females. TT_3 is associated with low energy availability and would give a better insight into the true energy availability state. Energy intake logs commonly underestimate food intake (underreporting issues), so it is hard to say with certainty that participants were in the energy state that was calculated. Furthermore, females that menstruate are at risk for iron deficiency. Low iron levels may induce low energy availability while low energy availability may contribute to iron deficiency. Thus, further investigation to dissect the causal relationship between iron deficiency and energy availability are needed.

Lastly, para-athlete populations need to be included in future research. Female para-athletes are a grossly understudied population of active females and only a few studies have investigated low energy availability in para-athletes and most were cross-sectional research that focused on national team athletes. Nonetheless, it has been suggested that para-athletes are at a greater risk for low energy availability compared to their able-bodied counterparts. Therefore, future longitudinal research in para-athletes is warranted.

REFERENCES

Acosta, F. M., Martinez-Tellez, B., Sanchez-Delgado, G., Migueles, J. H., Contreras-Gomez, M. A., Martinez-Avila, W. D., Merchan-Ramirez, E., Alcantara, J. M. A., Amaro-Gahete, F. J., Llamas-Elvira, J. M., & Ruiz, J. R. (2019). Association of objectively measured physical activity with brown adipose tissue volume and activity in young adults. *The Journal of Clinical Endocrinology & Metabolism*, *104*(2), 223–233. https://doi.org/10.1210/jc.2018-01312

Ainsworth, B. E., Haskell, W. L., Herrmann, S. D., Meckes, N., Bassett, D. R., Tudor-Locke, C., Greer, J. L., Vezina, J., Whitt-Glover, M. C., & Leon, A. S. (2011). 2011 Compendium of Physical Activities: A second update of codes and MET values. *Medicine and Science in Sports and Exercise*, *43*(8), 1575–1581. https://doi.org/10.1249/MSS.0b013e31821ece12

Ainsworth, B. E., Haskell, W. L., Whitt, M. C., Irwin, M. L., Swartz, A. M., Strath, S. J., O'Brien, W. L., Bassett, D. R., Schmitz, K. H., Emplaincourt, P. O., Jacobs, D. R., & Leon, A. S. (2000). Compendium of physical activities: An update of activity codes and MET intensities. *Medicine and Science in Sports and Exercise*, *32*(9 Suppl), S498-504. https://doi.org/10.1097/00005768-200009001-00009

Alfonso, C., & Capdevila, L. (2022). Heart rate variability, mood and performance: A pilot study on the interrelation of these variables in amateur road cyclists. *PeerJ*, *10*, e13094. https://doi.org/10.7717/peerj.13094

Allaway, H. C. M., Southmayd, E. A., & De Souza, M. J. (2016). The physiology of functional hypothalamic amenorrhea associated with energy deficiency in exercising women and in women with anorexia nervosa. *Hormone Molecular Biology and Clinical Investigation*, *25*(2). https://doi.org/10.1515/hmbci-2015-0053

Alvergne, A., & Tabor, V. H. (2018). Is female health cyclical? Evolutionary perspectives on menstruation. *Trends in Ecology & Evolution*, *33*(6), 399–414.

American Academy of Pediatrics, Committee on Adolescence, American College of Obstetricians and Gynecologists, & Committee on Adolescent Health Care. (2006). Menstruation in girls and adolescents: Using the menstrual cycle as a vital sign. *Pediatrics*, *118*(5), 2245–2250. https://doi.org/10.1542/peds.2006-2481

American Society for Reproductive Medicine and the Society for Reproductive Endocrinology and Infertility. (2021). Diagnosis and treatment of luteal phase deficiency: A committee opinion. *Fertility and Sterility*, *115*(6), 1416–1423. https://doi.org/10.1016/j.fertnstert.2021.02.010

Anai, T., Miyazaki, F., Tomiyasu, T., & Matsuo, T. (2001). Risk of irregular menstrual cycles and low peak bone mass during early adulthood associated with age at menarche. *Pediatrics International*, *43*(5), 483–488.

Andrews, M. A., Schliep, K. C., Wactawski-Wende, J., Stanford, J. B., Zarek, S. M., Radin, R. G., Sjaarda, L. A., Perkins, N. J., Kalwerisky, R. A., Hammoud, A. O., & Mumford, S. L. (2015). Dietary factors and luteal phase deficiency in healthy eumenorrheic women. *Human Reproduction*, *30*(8), 1942–1951. https://doi.org/10.1093/humrep/dev133

Arslan, R. C., Blake, K., Botzet, L., Bürkner, P.-C., DeBruine, L., Fiers, T., Grebe, N., Hahn, A., Jones, B. C., Marcinkowska, U. M., Mumford, S. L., Penke, L., Roney, J., Schisterman, E., & Stern, J. (2022). Not within spitting distance: Salivary immunoassays of estradiol have subpar validity for cycle phase. *PsyArXiv*. https://doi.org/10.31234/osf.io/5r8mg

Asarian, L., & Geary, N. (2006). Modulation of appetite by gonadal steroid hormones. *Philosophical Transactions of the Royal Society B: Biological Sciences*. https://doi.org/10.1098/rstb.2006.1860

Ayabe, T., Tsutsumi, O., Momoeda, M., Yano, T., Mitsuhashi, N., & Taketani, Y. (1994). Impaired follicular growth and abnormal luteinizing hormone surge in luteal phase defect. *Fertility and Sterility*, *61*(4), 652–656. https://doi.org/10.1016/S0015-0282(16)56641-2

Baggio, S., Di Paola, R., Zaffagnini, S., Fino, S., Raffaelli, R., & Franchi, M. (2019). Functional and endocrine-metabolic oligomenorrhea: Proposal of a new diagnostic assessment tool for differential diagnosis in adolescence. *Journal of Pediatric Endocrinology and Metabolism*, *32*(2), 135–142. https://doi.org/10.1515/jpem-2018-0351

Baker, E. R., Mathur, R. S., Kirk, R. F., & Williamson, H. O. (1981). Female runners and secondary amenorrhea: Correlation with age, parity, mileage, and plasma hormonal and sex-hormone-binding globulin concentrations. *Fertility and Sterility*, *36*(2), 183–187. https://doi.org/10.1016/S0015-0282(16)45676-1

Balasch, J., & Fábregues, F. (2002). Is luteinizing hormone needed for optimal ovulation induction? *Current Opinion in Obstetrics and Gynecology*, *14*(3), 265–274.

Balasch, J., & Fábregues, F. (2006). LH in the follicular phase: Neither too high nor too low. *Reproductive BioMedicine Online*, *12*(4), 406–415. https://doi.org/10.1016/S1472-6483(10)61991-8

Barr, S. I., Janelle, K. C., & Prior, J. C. (1995). Energy intakes are higher during the luteal phase of ovulatory menstrual cycles. *The American Journal of Clinical Nutrition*, *61*(1), 39–43. https://doi.org/10.1093/ajcn/61.1.39

Barrack, M. T., Gibbs, J. C., De Souza, M. J., Williams, N. I., Nichols, J. F., Rauh, M. J., & Nattiv, A. (2014). Higher incidence of bone stress injuries with increasing female athlete triad–related risk factors: A prospective multisite study of exercising girls and women. *The American Journal of Sports Medicine*, *42*(4), 949–958. https://doi.org/10.1177/0363546513520295

Beitins, I. Z., McARTHUR, J. W., Turnbull, B. A., Skrinar, G. S., & Bullen, B. A. (1991). Exercise induces two types of human luteal dysfunction: Confirmation by urinary free progesterone. *The Journal of Clinical Endocrinology & Metabolism*, *72*(6), 1350–1358. https://doi.org/10.1210/jcem-72-6-1350

Bellem, A., Meiyappan, S., Romans, S., & Einstein, G. (2011). Measuring estrogens and progestagens in humans: An overview of methods. *Gender Medicine*, *8*(5), 283–299. https://doi.org/10.1016/j.genm.2011.07.001

Benton, M. J., Hutchins, A. M., & Dawes, J. J. (2020). Effect of menstrual cycle on resting metabolism: A systematic review and meta-analysis. *PLOS ONE*, *15*(7), e0236025. https://doi.org/10.1371/journal.pone.0236025

Berbic, M., & Fraser, I. S. (2013). Immunology of Normal and Abnormal Menstruation. *Women's Health*, *9*(4), 387–395. https://doi.org/10.2217/WHE.13.32

Black, A. (2000). Critical evaluation of energy intake using the Goldberg cut-off for energy intake:basal metabolic rate. A practical guide to its calculation, use and limitations. *International Journal of Obesity*, *24*(9), 1119–1130. https://doi.org/10.1038/sj.ijo.0801376

Black, A. E. (2000). The sensitivity and specificity of the Goldberg cut-off for EI:BMR for identifying diet reports of poor validity. *European Journal of Clinical Nutrition*, *54*(5), Article 5. https://doi.org/10.1038/sj.ejcn.1600971

Black, K., Slater, J., Brown, R., & Cooke, R. (2018). Low energy availability, plasma lipids, and hormonal profiles of recreational athletes. *Journal of Strength and Conditioning Research*, *32*(10), 2816–2824. https://doi.org/10.1519/JSC.0000000000002540

Blundell, J. E., Gibbons, C., Beaulieu, K., Casanova, N., Duarte, C., Finlayson, G., Stubbs, R. J., & Hopkins, M. (2020). The drive to eat in homo sapiens: Energy expenditure drives energy intake. *Physiology & Behavior*, *219*. https://eprints.whiterose.ac.uk/157644/

Borg, G. (1998). *Borg's perceived exertion and pain scales* (pp. viii, 104). Human Kinetics.

Boutzios, G., Karalaki, M., & Zapanti, E. (2013). Common pathophysiological mechanisms involved in luteal phase deficiency and polycystic ovary syndrome. Impact on fertility. *Endocrine*, *43*(2), 314–317. https://doi.org/10.1007/s12020-012-9778-9

Boyd, N. F., Lockwood, G. A., Greenberg, C. V., Martin, L. J., & Tritchler, D. L. (1997). Effects of a low-fat high-carbohydrate diet on plasma sex hormones in premenopausal women: Results from a randomized controlled trial. Canadian Diet and Breast Cancer Prevention Study Group. *British Journal of Cancer*, *76*(1), 127–135.

Boyden, T. W., Pamenter, R. W., Stanforth, P. R., Rotkis, T. C., & Wilmore, J. H. (1984). Impaired gonadotropin responses to gonadotropin-releasing hormone stimulation in endurance-trained women. *Fertility and Sterility*, *41*(3), 359–363. https://doi.org/10.1016/S0015-0282(16)47711-3

Bronson, F. H. (1986). Food-restricted, prepubertal, female rats: Rapid recovery of luteinizing hormone pulsing with excess food, and full recovery of pubertal development with gonadotropin-releasing hormone. *Endocrinology*, *118*(6), 2483–2487. https://doi.org/10.1210/endo-118-6-2483

Broocks, A., Pirke, K. M., Schweiger, U., Tuschl, R. J., Laessle, R. G., Strowitzki, T., Horl, E., Horl, T., Haas, W., & Jeschke, D. (1990). Cyclic ovarian function in recreational athletes. *Journal of Applied Physiology*, *68*(5), 2083–2086. https://doi.org/10.1152/jappl.1990.68.5.2083

Buffenstein, R., Poppitt, S. D., McDevitt, R. M., & Prentice, A. M. (1995). Food intake and the menstrual cycle: A retrospective analysis, with implications for appetite research. *Physiology & Behavior*, *58*(6), 1067–1077. https://doi.org/10.1016/0031-9384(95)02003-9

Bukulmez, O., & Arici, A. (2004). Luteal phase defect: Myth or reality. *Obstetrics and Gynecology Clinics of North America*, *31*(4), 727–744. https://doi.org/10.1016/j.ogc.2004.08.007

Bull, F. C., Al-Ansari, S. S., Biddle, S., Borodulin, K., Buman, M. P., Cardon, G., Carty, C., Chaput, J.-P., Chastin, S., Chou, R., Dempsey, P. C., DiPietro, L., Ekelund, U., Firth, J., Friedenreich, C. M., Garcia, L., Gichu, M., Jago, R., Katzmarzyk, P. T., … Willumsen, J. F. (2020). World Health Organization 2020 guidelines on physical activity and sedentary behaviour. *British Journal of Sports Medicine*, *54*(24), 1451–1462. https://doi.org/10.1136/bjsports-2020-102955

Bullen, B. A., Skrinar, G. S., Beitins, I. Z., von Mering, G., Turnbull, B. A., & McArthur, J. W. (1985). Induction of menstrual disorders by strenuous exercise in untrained women. *New England Journal of Medicine*, *312*(21), 1349–1353. https://doi.org/10.1056/NEJM198505233122103

Bullough, R. C., Gillette, C. A., Harris, M. A., & Melby, C. L. (1995). Interaction of acute changes in exercise energy expenditure and energy intake on resting metabolic rate. *The American Journal of Clinical Nutrition*, *61*(3), 473–481. https://doi.org/10.1093/ajcn/61.3.473

Burke, L. M., Lundy, B., Fahrenholtz, I. L., & Melin, A. K. (2018). Pitfalls of conducting and interpreting estimates of energy availability in free-living athletes. *International Journal of Sport Nutrition and Exercise Metabolism*, *28*(4), 350–363. https://doi.org/10.1123/ijsnem.2018-0142

Burrows, M. (2003). Physiological factors associated with low bone mineral density in female endurance runners. *British Journal of Sports Medicine*, *37*(1), 67–71. https://doi.org/10.1136/bjsm.37.1.67

Byrne, N. M., Hills, A. P., Hunter, G. R., Weinsier, R. L., & Schutz, Y. (2005). Metabolic equivalent: One size does not fit all. *Journal of Applied Physiology*, *99*(3), 1112–1119. https://doi.org/10.1152/japplphysiol.00023.2004

Cadegiani, F. A., & Kater, C. E. (2017). Hormonal aspects of overtraining syndrome: A systematic review. *BMC Sports Science, Medicine and Rehabilitation*, *9*(1), 14. https://doi.org/10.1186/s13102-017-0079-8

Capling, L., Beck, K. L., Gifford, J. A., Slater, G., Flood, V. M., & O'Connor, H. (2017). Validity of dietary assessment in athletes: A systematic review. *Nutrients*, *9*(12), Article 12. https://doi.org/10.3390/nu9121313

Chabbert-Buffet, N., & Bouchard, P. (2002). The normal human menstrual cycle. *Reviews in Endocrine & Metabolic Disorders*, *3*(3), 173–183.

Chappell, S., & Hackney, A. C. (1997). Associations between menstrual cycle phase, physical activity level, and dietary macronutrient intake. *Biology of Sport*, *14*(4), 251–258.

Chatterton, R. T., Haan, J. N., Jenco, J. M., & Cheesman, K. L. (1982). Radioimmunoassay of pregnanediol concentrations in early morning urine specimens for assessment of luteal function in women. *Fertility and Sterility*, *37*(3), 361–366. https://doi.org/10.1016/S0015-0282(16)46096-6

Chavarro, J. E., Rich-Edwards, J. W., Rosner, B. A., & Willett, W. C. (2007). Diet and lifestyle in the prevention of ovulatory disorder infertility. *Obstetrics & Gynecology*, *110*(5), 1050–1058. https://doi.org/10.1097/01.AOG.0000287293.25465.e1

Cialdella-Kam, L., Guebels, C. P., Maddalozzo, G. F., & Manore, M. M. (2014). Dietary intervention restored menses in female athletes with exercise-associated menstrual dysfunction with limited impact on bone and muscle health. *Nutrients*, *6*(8). https://doi.org/10.3390/nu6083018

Cockerill, I. M., Nevill, A. M., & Byrne, N. C. (1992). Mood, mileage and the menstrual cycle. *British Journal of Sports Medicine*, *26*(3), 145–150. https://doi.org/10.1136/bjsm.26.3.145

Codner, E., Eyzaguirre, F. C., Iñiguez, G., López, P., Pérez-Bravo, F., Torrealba, I. M., & Cassorla, F. (2011). Ovulation rate in adolescents with type 1 diabetes mellitus. *Fertility and Sterility*, *95*(1), 197-202.e1. https://doi.org/10.1016/j.fertnstert.2010.10.041

Codner, E., Villarroel, C., Eyzaguirre, F. C., López, P., Merino, P. M., Pérez-Bravo, F., Iñiguez, G., & Cassorla, F. (2011). Polycystic ovarian morphology in postmenarchal adolescents. *Fertility and Sterility*, *95*(2), 702-706.e2. https://doi.org/10.1016/j.fertnstert.2010.06.015

Cokkinades, V. E., Macera, C. A., & Pate, R. R. (1990). Menstrual dysfunction among habitual runners. *Women & Health*, *16*(2), 59–69. https://doi.org/10.1300/J013v16n02_05

Collette, R., Kellmann, M., Ferrauti, A., Meyer, T., & Pfeiffer, M. (2018). Relation between training load and recovery-stress state in high-performance swimming. *Frontiers in Physiology*, *9*. https://www.frontiersin.org/articles/10.3389/fphys.2018.00845

Corr, M., De Souza, M. J., Toombs, R. J., & Williams, N. I. (2011). Circulating leptin concentrations do not distinguish menstrual status in exercising women. *Human Reproduction*, *26*(3), 685–694. https://doi.org/10.1093/humrep/deq375

Cumming, D. C., Vickovic, M. M., Wall, S. R., & Fluker, M. R. (1985). Defects in pulsatile LH release in normally menstruating runners. *The Journal of Clinical Endocrinology & Metabolism*, *60*(4), 810–812. https://doi.org/10.1210/jcem-60-4-810

Dale, E., Gerlach, D. H., & Wilhite, A. L. (1979). Menstrual dysfunction in distance runners. *Obstetrics and Gynecology*, *54*(1), 47–53. https://doi.org/10.1097/00006250-197907000-00013

Day, D. S., Gozansky, W. S., Van Pelt, R. E., Schwartz, R. S., & Kohrt, W. M. (2005). Sex hormone suppression reduces resting energy expenditure and β-adrenergic support of resting energy expenditure. *The Journal of Clinical Endocrinology & Metabolism*, *90*(6), 3312–3317. https://doi.org/10.1210/jc.2004-1344

de Jonge, X. A. K. J. (2003). Effects of the menstrual cycle on exercise performance. *Sports Medicine*, *33*(11), 833–851. https://doi.org/10.2165/00007256-200333110-00004

De Souza, M. J. (2003). Menstrual disturbances in athletes: A focus on luteal phase defects: *Medicine & Science in Sports & Exercise*, *35*(9), 1553–1563. https://doi.org/10.1249/01.MSS.0000084530.31478.DF

De Souza, M. J., Koltun, K., Etter, C., & Ricker, E. (2017). Current status of the female athlete triad: Update and future directions. *Current Osteoporosis Reports*, *15*. https://doi.org/10.1007/s11914-017-0412-x

De Souza, M. J., Koltun, K. J., Strock, N. C., & Williams, N. I. (2019). Rethinking the concept of an energy availability threshold and its role in the Female Athlete Triad. *Current Opinion in Physiology*, *10*, 35–42. https://doi.org/10.1016/j.cophys.2019.04.001

De Souza, M. J., Lee, D. K., VanHeest, J. L., Scheid, J. L., West, S. L., & Williams, N. I. (2007). Severity of energy-related menstrual disturbances increases in proportion to indices of energy conservation in exercising women. *Fertility and Sterility*, *88*(4), 971–975. https://doi.org/10.1016/j.fertnstert.2006.11.171

De Souza, M. J., Leidy, H. J., O'Donnell, E., Lasley, B. L., & Williams, N. I. (2004). Fasting Ghrelin Levels in Physically Active Women: Relationship with Menstrual Disturbances and Metabolic Hormones. *Journal of Clinical Endocrinology and Metabolism, 89*(7), 3536–3542.

De Souza, M. J., Mallinson, R. J., Strock, N. C. A., Koltun, K. J., Olmsted, M. P., Ricker, E. A., Scheid, J. L., Allaway, H. C., Mallinson, D. J., Kuruppumullage Don, P., & Williams, N. I. (2021). Randomised controlled trial of the effects of increased energy intake on menstrual recovery in exercising women with menstrual disturbances: The 'REFUEL' study. *Human Reproduction*, *36*(8), 2285–2297. https://doi.org/10.1093/humrep/deab149

De Souza, M. J., Miller, B. E., Loucks, A. B., Luciano, A. A., Pescatello, L. S., Campbell, C. G., & Lasley, B. L. (1998a). High frequency of luteal phase deficiency and anovulation in recreational women runners: Blunted elevation in follicle-stimulating hormone observed during luteal-follicular transitio*n*. *Journal of Clinical Endocrinology and Metabolism* *83*(12), 13.

De Souza, M. J., Miller, B. E., Loucks, A. B., Luciano, A. A., Pescatello, L. S., Campbell, C. G., & Lasley, B. L. (1998b). High frequency of luteal phase deficiency and anovulation in recreational women runners: Blunted elevation in follicle-stimulating hormone observed during luteal-follicular transition. *The Journal of Clinical Endocrinology and Metabolism*, *83*(12), 4220–4232. https://doi.org/10.1210/jcem.83.12.5334

De Souza, M. J., Miller, B. E., Sequenzia, L. C., Luciano, A. A., Ulreich, S., Stier, S., Prestwood, K., & Lasley, B. L. (1997). Bone health is not affected by luteal phase abnormalities and decreased ovarian progesterone production in female runners. *The Journal of Clinical Endocrinology & Metabolism*, *82*(9), 2867–2876. https://doi.org/10.1210/jcem.82.9.4201

De Souza, M. J., Toombs, R. J., Scheid, J. L., O'Donnell, E., West, S. L., & Williams, N. I. (2010a). High prevalence of subtle and severe menstrual disturbances in exercising women: Confirmation using daily hormone measures. *Human Reproduction*, *25*(2), 491–503. https://doi.org/10.1093/humrep/dep411

De Souza, M. J., Toombs, R. J., Scheid, J. L., O'Donnell, E., West, S. L., & Williams, N. I. (2010b). High prevalence of subtle and severe menstrual disturbances in exercising women: Confirmation using daily hormone measures. *Human Reproduction*, *25*(2), 491–503. https://doi.org/10.1093/humrep/dep411

De Souza, M. J., Van Heest, J., Demers, L. M., & Lasley, B. L. (2003). Luteal phase deficiency in recreational runners: Evidence for a hypometabolic state. *The Journal of Clinical Endocrinology and Metabolism*, *88*(1), 337–346. https://doi.org/10.1210/jc.2002-020958

De Souza, M. J., West, S. L., Jamal, S. A., Hawker, G. A., Gundberg, C. M., & Williams, N. I. (2008). The presence of both an energy deficiency and estrogen deficiency exacerbate alterations of bone metabolism in exercising women. *Bone*, *43*(1), 140–148. https://doi.org/10.1016/j.bone.2008.03.013

De Souza, M. J., & Williams, N. I. (2004). Physiological aspects and clinical sequelae of energy deficiency and hypoestrogenism in exercising women. *Human Reproduction Update, 10*(5), 433–448.

De Souza, M. J., & Williams, N. I. (2005). Beyond hypoestrogenism in amenorrheic athletes: energy deficiency as a contributing factor for bone loss. *Current Sports Medicine Reports*, *4*(1), 38–44. https://doi.org/10.1097/01.CSMR.0000306070.67390.cb

Derry, G. N., & Derry, P. S. (2010). Characterization of chaotic dynamics in the human menstrual cycle. *Nonlinear Biomedical Physics*, *4*(1), 5.

Dietary Guidelines for Americans, 2020-2025. (n.d.).

Ding, D., Mielke, G. I., Silva, I. C. M., Wehrmeister, F. C., Horta, B. L., Brage, S., Hallal, P. C., & Ekelund, U. (2020). Prenatal and birth predictors of objectively measured physical activity and sedentary time in three population-based birth cohorts in Brazil. *Scientific Reports*, *10*(1), Article 1. https://doi.org/10.1038/s41598-019-57070-x

Dragano, N., Milbank, E., & López, M. (2020). Estradiol and appetite: To eat or not to eat. *Molecular Metabolism*, *42*, 101061. https://doi.org/10.1016/j.molmet.2020.101061

Elliott, S. A., Ng, J., Leow, M. K.-S., & Henry, C. J. K. (2015). The influence of the menstrual cycle on energy balance and taste preference in Asian Chinese women. *European Journal of Nutrition*, *54*(8), 1323–1332. https://doi.org/10.1007/s00394-014-0812-y

Elliott-Sale, K. J., McNulty, K. L., Ansdell, P., Goodall, S., Hicks, K. M., Thomas, K., Swinton, P. A., & Dolan, E. (2020). The effects of oral contraceptives on exercise performance in women: A systematic review and meta-analysis. *Sports Medicine*, *50*(10), 1785–1812. https://doi.org/10.1007/s40279-020-01317-5

Elliott-Sale, K. J., Minahan, C. L., de Jonge, X. A. K. J., Ackerman, K. E., Sipilä, S., Constantini, N. W., Lebrun, C. M., & Hackney, A. C. (2021). Methodological considerations for studies in sport and exercise science with women as participants: A working guide for standards of practice for research on women. *Sports Medicine*, *51*(5), 843–861. https://doi.org/10.1007/s40279-021-01435-8

Ellison, P. T. (1988). Human salivary steroids: Methodological considerations and applications in physical anthropology. *American Journal of Physical Anthropology*, *31*(S9), 115–142. https://doi.org/10.1002/ajpa.1330310507

Ellison, P. T. (1993). Measurements of salivary progesterone. *Annals of the New York Academy of Sciences*, *694*(1), 161–175. https://doi.org/10.1111/j.1749-6632.1993.tb18350.x

Ellison, P. T., Lager, C., & Calfee, J. (1987). Low profiles of salivary progesterone among college undergraduate women. *Journal of Adolescent Health Care: Official Publication of the Society for Adolescent Medicine*, *8*(2), 204–207. https://doi.org/10.1016/0197-0070(87)90266-x

Evans, J. J. (1986). Progesterone in saliva does not parallel unbound progesterone in plasma. *Clinical Chemistry*, *32*(3), 542–544. https://doi.org/10.1093/clinchem/32.3.542

Evenepoel, C., Clevers, E., Deroover, L., Loo, W. V., Matthys, C., & Verbeke, K. (2020). Accuracy of nutrient calculations using the consumer-focused online app MyFitnessPal: Validation study. *Journal of Medical Internet Research*, *22*(10), e18237. https://doi.org/10.2196/18237

Evenepoel, C., Clevers, E., Deroover, L., Matthys, C., & Verbeke, K. (2020). Dietary assessment with the online platform MyFitnessPal: A reliable method? *Proceedings of the Nutrition Society*, *79*(OCE2). https://doi.org/10.1017/S0029665120004541

Fahrenholtz, I. L., Sjodin, A., Bernardot, Tornberg, Skouby, Faber, Sundgot-Borgen, & Melin, A. (2018). Within-day energy deficiency and reproductive function in female endurance athletes. *Scan J Med Sci Sports, 28*(3).

Feicht, C. B., Johnson, T. S., Martin, B. J., Sparkes, K. E., & Wagner, W. W. (1978). Secondary amenorrhea in athletes. *The Lancet*, *312*(8100), 1145–1146. https://doi.org/10.1016/S0140-6736(78)92294-8

Filicori, M., Butler, J. P., & Crowley, W. F. (1984). Neuroendocrine regulation of the corpus luteum in the human. Evidence for pulsatile progesterone secretion. *The Journal of Clinical Investigation*, *73*(6), 1638–1647. https://doi.org/10.1172/JCI111370

Fisher, E. C., Nelson, M. E., Frontera, W. R., Turksoy, R. N., & Evans, W. J. (1986). Bone mineral content and levels of gonadotropins and estrogens in amenorrheic running women. *The Journal of Clinical Endocrinology & Metabolism*, *62*(6), 1232–1236. https://doi.org/10.1210/jcem-62-6-1232

Folscher, L.-L., Grant, C. C., Fletcher, L., & Janse van Rensberg, D. C. (2015). Ultra-Marathon athletes at risk for the female athlete triad. *Sports Medicine - Open*, *1*(1), 29. https://doi.org/10.1186/s40798-015-0027-7

Franklin, R. J. M., & ffrench-Constant, C. (2008). Remyelination in the CNS: From biology to therapy. *Nature Reviews Neuroscience*, *9*(11), 839–855. https://doi.org/10.1038/nrn2480

Franz, W. B. (1988). Basic Review: Endocrinology of the Normal Menstrual Cycle. *Primary Care: Clinics in Office Practice*, *15*(3), 607–616. https://doi.org/10.1016/S0095-4543(21)01279-3

Friday, E., & Drinkwater, L. (1993). Elevated Plasma Low-Density Lipoprotein and High- Density Lipoprotein Cholesterol Levels in Amenorrheic Athletes: Effects of Endogenous Hormone Status and Nutrient Intake. *J Clin Endocrinol Metab, 77*(6), 5.

Frisch, R. E. (1981). Delayed menarche and amenorrhea of college athletes in relation to age of onset of training. *JAMA: The Journal of the American Medical Association*, *246*(14), 1559. https://doi.org/10.1001/jama.1981.03320140047029

Frisch, R. E., Wyshak, G., & Vincent, L. (1980). Delayed menarche and amenorrhea in ballet dancers. *New England Journal of Medicine*, *303*(1), 17–19. https://doi.org/10.1056/NEJM198007033030105

Gandara, B. K., Leresche, L., & Mancl, L. (2007). Patterns of salivary estradiol and progesterone across the menstrual cycle. *Annals of the New York Academy of Sciences*, *1098*(1), 446–450. https://doi.org/10.1196/annals.1384.022

Gaskins, A. J., Mumford, S. L., Wactawski-Wende, J., & Schisterman, E. F. (2012). Effect of daily fiber intake on luteinizing hormone levels in reproductive-aged women. *European Journal of Nutrition*, *51*(2), 249–253. https://doi.org/10.1007/s00394-011-0207-2

Gaskins, A. J., Mumford, S. L., Zhang, C., Wactawski-Wende, J., Hovey, K. M., Whitcomb, B., Howards, P., Perkins, N. J., Yeung, E. H., & Schisterman, E. F. (2009). Effect of daily fiber intake on reproductive function: The BioCycle Study. *The American Journal of Clinical Nutrition*, *90*(4), 1061–1069. https://doi.org/10.3945/ajcn.2009.27990

Gibbs, J. C., Nattiv, A., Barrack, M. T., Williams, N. I., Rauh, M. J., Nichols, J. F., & De Souza, M. J. (2014). Low bone density risk is higher in exercising women with multiple triad risk factors. *Medicine & Science in Sports & Exercise*, *46*(1), 167–176. https://doi.org/10.1249/MSS.0b013e3182a03b8b

Gibbs, J. C., Williams, N. I., & De Souza, M. J. (2013). Prevalence of individual and combined components of the female athlete triad. *Medicine & Science in Sports & Exercise*, *45*(5), 985–996. https://doi.org/10.1249/MSS.0b013e31827e1bdc

Giersch, G. E. W., Charkoudian, N., Morrissey, M. C., Butler, C. R., Colburn, A. T., Caldwell, A. R., Kavouras, S. A., & Casa, D. J. (2021). Estrogen to progesterone ratio and fluid regulatory responses to varying degrees and methods of dehydration. *Frontiers in Sports and Active Living*, *3*. https://www.frontiersin.org/articles/10.3389/fspor.2021.722305

Goldberg, G. R., Black, A. E., Jebb, S. A., Cole, T. J., Murgatroyd, P. R., Coward, W. A., & Prentice, A. M. (1991). Critical evaluation of energy intake data using fundamental principles of energy physiology: 1. Derivation of cut-off limits to identify under-recording. *European Journal of Clinical Nutrition*, *45*(12), 569–581.

Goodale, B. M., Shilaih, M., Falco, L., Dammeier, F., Hamvas, G., & Leeners, B. (2019). Wearable sensors reveal menses-driven changes in physiology and enable prediction of the fertile window: Observational study. *Journal of Medical Internet Research*, *21*(4), e13404. https://doi.org/10.2196/13404

Gordon, C. M., Ackerman, K. E., Berga, S. L., Kaplan, J. R., Mastorakos, G., Misra, M., Murad, M. H., Santoro, N. F., & Warren, M. P. (2017). Functional hypothalamic amenorrhea: An Endocrine Society Clinical Practice Guideline. *The Journal of Clinical Endocrinology & Metabolism*, *102*(5), 1413–1439. https://doi.org/10.1210/jc.2017-00131

Grant, L. K., Gooley, J. J., St Hilaire, M. A., Rajaratnam, S. M. W., Brainard, G. C., Czeisler, C. A., Lockley, S. W., & Rahman, S. A. (2020). Menstrual phase-dependent differences in neurobehavioral performance: The role of temperature and the progesterone/estradiol ratio. *Sleep*, *43*(2), zsz227. https://doi.org/10.1093/sleep/zsz227

Gray, D. P., & Dale, E. (1983). Variables associated with secondary amenorrhea in women runners. *Journal of Sports Sciences*, *1*(1), 55–67. https://doi.org/10.1080/02640418308729659

Green, B. B., Daling, J. R., Weiss, N. S., Liff, J. M., & Koepsell, T. (1986). Exercise as a risk factor for infertility with ovulatory dysfunction. *American Journal of Public Health*, *76*(12), 1432–1436.

Greil, A. L., McQuillan, J., Lowry, M., & Shreffler, K. M. (2011). Infertility treatment and fertility-specific distress: A longitudinal analysis of a population-based sample of U.S. women. *Social Science & Medicine*, *73*(1), 87–94. https://doi.org/10.1016/j.socscimed.2011.04.023

Grunfeld, L., Sandler, B., Fox, J., Boyd, C., Kaplan, P., & Navot, D. (1989). Luteal phase deficiency after completely normal follicular and periovulatory phases. *Fertility and Sterility*, *52*(6), 919–923. https://doi.org/10.1016/S0015-0282(16)53152-5

Gudgeon, B., Bchir, M., & Howard, B. (1990). Evaluation of the accuracy of the home ovulation detection kit, Clearplan, at predicting ovulation. *Medical Journal of Australia.*

Guebels, C. P., Kam, L. C., Maddalozzo, G. F., & Manore, M. M. (2014). Active women before/after an intervention designed to restore menstrual function: Resting metabolic rate and comparison of four methods to quantify energy expenditure and energy availability. *International Journal of Sport Nutrition and Exercise Metabolism*, *24*(1), 37–46. https://doi.org/10.1123/ijsnem.2012-0165

Guermandi, E., Vegetti, W., Bianchi, M. M., Uglietti, A., Ragni, G., & Crosignani, P. (2001). Reliability of ovulation tests in infertile women. *Obstetrics & Gynecology*, *97*(1), 92–96. https://doi.org/10.1016/S0029-7844(00)01083-8

Hackney, A. C., & Constantini, N. W. (2020). *Endocrinology of Physical Activity and Sport*. Springer Nature.

Hamilton-Fairley, D. (2003). Anovulation. *BMJ*, *327*(7414), 546–549. https://doi.org/10.1136/bmj.327.7414.546

Harmon, Q., Kissell, K. A., Jukic, A. M. Z., Sjaarda, L. A., Perkins, N. J., Umbach, D. M., Schisterman, E. F., Baird, D. D., & Mumford, S. L. (2020). Vitamin D and Reproductive Hormones Across the Menstrual Cycle. *Human Reproduction, 35*(2), 413–423.

Hayes, A. F. (2012). PROCESS: A Versatile Computational Tool for Observed Variable Mediation, Moderation, and Conditional Process Modeling. http://www.afhayes.com/public/process2012.pdf

Hayes, M., Chustek, M., Wang, Z., Gallagher, D., Heshka, S., Spungen, A., Bauman, W., & Heymsfield, S. B. (2002). DXA: Potential for creating a metabolic map of organ-tissue resting energy expenditure components. *Obesity Research*, *10*(10), 969–977. https://doi.org/10.1038/oby.2002.132

Hees, V. T. van, Gorzelniak, L., León, E. C. D., Eder, M., Pias, M., Taherian, S., Ekelund, U., Renström, F., Franks, P. W., Horsch, A., & Brage, S. (2013). Separating movement and

gravity components in an acceleration signal and implications for the assessment of human daily physical activity. *PLOS ONE*, *8*(4), e61691. https://doi.org/10.1371/journal.pone.0061691

Henry, C. J. K., Lightowler, H. J., & Marchini, J. (2003). Intra-individual variation in resting metabolic rate during the menstrual cycle. *British Journal of Nutrition*, *89*(6), 811–817. https://doi.org/10.1079/BJN2003839

Hetland, M. L., Haarbo, J., & Christiansen, C. (1995). Body composition and serum lipids in female runners: Influence of exercise level and menstrual bleeding pattern. *European Journal of Clinical Investigation*, *25*(8), 553–558. https://doi.org/10.1111/j.1365-2362.1995.tb01745.x

Hilton, L., & Loucks, A. B. (2000). Low energy availability, not exercise stress, suppresses the diurnal rhythm of leptin in healthy young women. *American Journal of Physiology-Endocrinology and Metabolism.*

Hirschberg, A. L. (2012). Sex hormones, appetite and eating behaviour in women. *Maturitas*, *71*(3), 248–256. https://doi.org/10.1016/j.maturitas.2011.12.016

Howe, J. C., Rumpler, W. V., & Seale, J. L. (1993). Energy expenditure by indirect calorimetry in premenopausal women: Variation within one menstrual cycle. *The Journal of Nutritional Biochemistry*, *4*(5), 268–273. https://doi.org/10.1016/0955-2863(93)90096-F

Hull, M. G. R., Savage, P. E., Bromham, D. R., Ismail, A. A. A., & Morris, A. F. (1982). The value of a single serum progesterone measurement in the midluteal phase as a criterion of a potentially fertile cycle (“ovulation”) derived from treated and untreated conception cycles. *Fertility and Sterility*, *37*(3), 355–360. https://doi.org/10.1016/S0015-0282(16)46095-4

Ihalainen, J. K., Löfberg, I., Kotkajuuri, A., Kyröläinen, H., Hackney, A. C., Taipale-Mikkonen, R. S. (2021). Influence of Menstrual Cycle or Hormonal Contraceptive Phase on Energy Intake and Metabolic Hormones—A Pilot Study. *Endocrines*, *2*(2), 79. http://dx.doi.org/10.3390/endocrines2020008

Ishikawa, M., Sengoku, K., Tamate, K., Takaoka, Y., Kane, M., & Fottrell, P. F. (2002). The clinical usefulness of salivary progesterone measurement for the evaluation of the corpus luteum function. *Gynecologic and Obstetric Investigation*, *53*(1), 32–37. https://doi.org/10.1159/000049408

Israel, R., Mishell, D. R., Stone, S. C., Thorneycroft, I. H., & Moyer, D. L. (1972). Single luteal phase serum progesterone assay as an indicator of ovulation. *American Journal of Obstetrics and Gynecology*, *112*(8), 1043–1046. https://doi.org/10.1016/0002-9378(72)90178-0

Janse De Jonge, X., Thompson, B., & Han, A. (2019). Methodological recommendations for menstrual cycle research in sports and exercise. *Medicine & Science in Sports & Exercise*, *51*(12), 2610–2617. https://doi.org/10.1249/MSS.0000000000002073

Jasienska, G. (2003). Energy metabolism and the evolution of reproductive suppression in the human female. *Acta Biotheoretica*, *51*(1), 1–18. http://dx.doi.org/10.1023/A:1023035321162

Jasienska, G., Ziomkiewicz, A., Thune, I., Lipson, S. F., & Ellison, P. T. (2006). Habitual physical activity and estradiol levels in women of reproductive age. *European Journal of Cancer Prevention*, *15*(5), 439–445.

Johnson, S., Weddell, S., Godbert, S., Freundl, G., Roos, J., & Gnoth, C. (2015). Development of the first urinary reproductive hormone ranges referenced to independently determined

ovulation day. *Clinical Chemistry and Laboratory Medicine*, *0*. https://doi.org/10.1515/cclm-2014-1087

Jordan, J., Craig, K., Clifton, D. K., & Soules, M. R. (1994). Luteal phase defect: The sensitivity and specificity of diagnostic methods in common clinical use. *Fertility and Sterility*, *62*(1), 54–62. https://doi.org/10.1016/S0015-0282(16)56815-0

Jordan, S. L., Albracht-Schulte, K., & Robert-McComb, J. J. (2020). Micronutrient deficiency in athletes and inefficiency of supplementation: Is low energy availability a culprit? *PharmaNutrition*, *14*, 100229. https://doi.org/10.1016/j.phanu.2020.100229

Kang, J., Ratamess, N. A., Faigenbaurm, A. D., Bush, J. A., Roser, C., Montemarano, D., Mercado, H., Choma, M., Mendez, C., & Pollock, M. (2020). Use of heart rate index to predict oxygen uptake – A validation study. *International Journal of Exercise Science*, *13*(7), 1705–1717.

Kellmann, M., & Kolling, S. (2019). *The Acute Recovery and Stress Scale | 4 | Recovery and Stress in Sport*. Taylor and Francis. http://www.taylorfrancis.com/chapters/mono/10.4324/9780429423857-4/acute-recovery-stress-scale-michael-kellmann-sarah-k%C3%B6lling

Kesner, J. S., Wright, D. M., Schrader, S. M., Chin, N. W., & Krieg, E. F. (1992). Methods of monitoring menstrual function in field studies: Efficacy of methods. *Reproductive Toxicology*, *6*(5), 385–400. https://doi.org/10.1016/0890-6238(92)90002-B

Kim, K., Mills, J. L., Michels, K. A., Chaljub, E. N., Wactawski-Wende, J., Plowden, T. C., & Mumford, S. L. (2020). Dietary intakes of Vitamin B-2 (Riboflavin), Vitamin B-6, and Vitamin B-12 and ovarian cycle function among premenopausal women. *Journal of the Academy of Nutrition and Dietetics*, *120*(5), 885–892. https://doi.org/10.1016/j.jand.2019.10.013

Kim, K., Wactawski-Wende, J., Michels, K. A., Plowden, T. C., Chaljub, E. N., Sjaarda, L. A., & Mumford, S. L. (2017). Dairy food intake is associated with reproductive hormones and sporadic anovulation among healthy premenopausal women. *The Journal of Nutrition*, *147*(2), 218–226. https://doi.org/10.3945/jn.116.241521

Kim, Y. J., Tamadon, A., Park, H. T., Kim, H., & Ku, S.-Y. (2016). The role of sex steroid hormones in the pathophysiology and treatment of sarcopenia. *Osteoporosis and Sarcopenia*, *2*(3), 140–155. https://doi.org/10.1016/j.afos.2016.06.002

Klein, D. A., Paradise, S. L., & Reeder, R. M. (2019). Amenorrhea: A systematic approach to diagnosis and management. *American Family Physician*, *100*(1), 39–48.

Koehler, K., Williams, N. I., Mallinson, R. J., Southmayd, E. A., Allaway, H. C. M., & De Souza, M. J. (2016). Low resting metabolic rate in exercise-associated amenorrhea is not due to a reduced proportion of highly active metabolic tissue compartments. *Am J Physiol Endocrinol Metab*, *311*. https://doi.org/10.1152/ajpendo.00110.2016

Kölling, S., Hitzschke, B., Holst, T., Ferrauti, A., Meyer, T., Pfeiffer, M., & Kellmann, M. (2015). Validity of the acute recovery and stress scale: Training monitoring of the German junior national field hockey team. *International Journal of Sports Science & Coaching*, *10*(2–3), 529–542. https://doi.org/10.1260/1747-9541.10.2-3.529

Kölling, S., Schaffran, P., Bibbey, A., Drew, M., Raysmith, B., Nässi, A., & Kellmann, M. (2020). Validation of the Acute Recovery and Stress Scale (ARSS) and the Short Recovery and Stress Scale (SRSS) in three English-speaking regions. *Journal of Sports Sciences*, *38*(2), 130–139. https://doi.org/10.1080/02640414.2019.1684790

Koltun, K. J., Williams, N. I., Scheid, J. L., & De Souza, M. J. (2020). Discriminating hypothalamic oligomenorrhea/amenorrhea from hyperandrogenic

oligomenorrhea/amenorrhea in exercising women. *Applied Physiology, Nutrition, and Metabolism*, *45*(7), 707–714. https://doi.org/10.1139/apnm-2019-0640

Kozey, S., Lyden, K., Staudenmayer, J., & Freedson, P. (2010). Errors in MET Estimates of Physical Activities Using 3.5 ml·kg−1·min−1 as the Baseline Oxygen Consumption. *Journal of Physical Activity and Health*, *7*(4), 508–516. https://doi.org/10.1123/jpah.7.4.508

Lai, J. D., Fantus, R. J., Cohen, A. J., Wan, V., Hudnall, M. T., Pham, M., Brannigan, R. E., & Halpern, J. A. (2021). Unmet financial burden of infertility care and the impact of state insurance mandates in the United States: Analysis from a popular crowdfunding platform. *Fertility and Sterility*, *116*(4), 1119–1125. https://doi.org/10.1016/j.fertnstert.2021.05.111

Landgren, B.-M., Undén, A.-L., & Diczfalusy, E. (1980). Hormonal profile of the cycle in 68 normally menstruating women. *Acta Endocrinologica*, *94*(1), 89–98. https://doi.org/10.1530/acta.0.0940089

Laughlin, G. A., & Yen, S. S. C. (1996). Nutritional and endocrine-metabolic aberrations in amenorrheic athletes*. The Journal of Clinical Endocrinology & Metabolism, 82*(12).

Lehmann, M., Knizia, K., Gastmann, U., Petersen, K. G., Khalaf, A. N., Bauer, S., Kerp, L., & Keul, J. (1993). Influence of 6-week, 6 days per week, training on pituitary function in recreational athletes. *British Journal of Sports Medicine*, *27*(3), 186–192. https://doi.org/10.1136/bjsm.27.3.186

Leiva, R., Burhan, U., Kyrillos, E., Fehring, R., McLaren, R., Dalzell, C., & Tanguay, E. (2014). Use of ovulation predictor kits as adjuncts when using fertility awareness methods (FAMs): A pilot study. *The Journal of the American Board of Family Medicine*, *27*(3), 427–429. https://doi.org/10.3122/jabfm.2014.03.130255

Levi-Setti, P. E., Cavagna, M., Baggiani, A., Zannoni, E., Colombo, G. V., & Liprandi, V. (2004). FSH and LH together in ovarian stimulation. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, *115*, S34–S39. https://doi.org/10.1016/j.ejogrb.2004.01.013

Li, R. H. W., & Ng, E. H. Y. (2012). Management of anovulatory infertility. *Best Practice & Research Clinical Obstetrics & Gynaecology*, *26*(6), 757–768. https://doi.org/10.1016/j.bpobgyn.2012.05.004

Lieberman, J. L., De Souza, M. J., Wagstaff, D. A., & Williams, N. I. (2018). Menstrual disruption with exercise is not linked to an energy availability threshold. *Medicine and Science in Sports and Exercise*, *50*(3), 551–561. https://doi.org/10.1249/MSS.0000000000001451

Lissner, L., Habicht, J. P., Strupp, B. J., Levitsky, D. A., Haas, J. D., & Roe, D. A. (1989). Body composition and energy intake: Do overweight women overeat and underreport? *The American Journal of Clinical Nutrition*, *49*(2), 320–325. https://doi.org/10.1093/ajcn/49.2.320

Liu, Y., Gold, E. B., Lasley, B. L., & Johnson, W. O. (2004). Factors Affecting Menstrual Cycle Characteristics. *American Journal of Epidemiology*, *160*(2), 131–140. https://doi.org/10.1093/aje/kwh188

Logue, D. M., Madigan, S. M., Heinen, M., McDonnell, S.-J., Delahunt, E., & Corish, C. A. (2019). Screening for risk of low energy availability in athletic and recreationally active females in Ireland. *European Journal of Sport Science*, *19*(1), 112–122. https://doi.org/10.1080/17461391.2018.1526973

López, M., & Tena-Sempere, M. (2015). Estrogens and the control of energy homeostasis: A brain perspective. *Trends in Endocrinology & Metabolism*, *26*(8), 411–421. https://doi.org/10.1016/j.tem.2015.06.003

Loucks, A. B. (2003). Energy availability, not body fatness, regulates reproductive function in women. *Exercise and Sport Sciences Reviews*, *31*(3), 144–148.

Loucks, A. B., & Horvath, S. M. (1984). Exercise-induced stress responses of amenorrheic and eumenorrheic runners. *The Journal of Clinical Endocrinology & Metabolism*, *59*(6), 1109–1120. https://doi.org/10.1210/jcem-59-6-1109

Loucks, A. B., & Horvath, S. M. (1985). Athletic amenorrhea: A review. *Medicine & Science in Sports & Exercise*, *17*(1), 56–72.

Loucks, A. B., Mortola, J. F., Girton, L., & Yen, S. S. C. (1989). Alterations in the hypothalamic-pituitary-ovarian and the hypothalamic-pituitary-adrenal axes in athletic women. *The Journal of Clinical Endocrinology & Metabolism*, *68*(2), 402–411. https://doi.org/10.1210/jcem-68-2-402

Loucks, A. B., & Thuma, J. R. (2003). Luteinizing hormone pulsatility is disrupted at a threshold of energy availability in regularly menstruating women. *The Journal of Clinical Endocrinology & Metabolism*, *88*(1), 297–311. https://doi.org/10.1210/jc.2002-020369

Loucks, A. B., Verdun, M., & Heath, E. M. (1998). Low energy availability, not stress of exercise, alters LH pulsatility in exercising women*. Journal of Applied Physiolog,*. *84*(1).

Lu, Y., Bentley, G. R., Gann, P. H., Hodges, K. R., & Chatterton, R. T. (1999). Salivary estradiol and progesterone levels in conception and nonconception cycles in women: Evaluation of a new assay for salivary estradiol. *Fertility and Sterility*, *71*(5), 863–868. https://doi.org/10.1016/S0015-0282(99)00093-X

Lutter, J. M., & Cushman, S. (1982). Menstrual patterns in female runners. *The Physician and Sportsmedicine*, *10*(9), 60–72. https://doi.org/10.1080/00913847.1982.11947320

Marshall, J. C., Dalkin, A. C., Haisenleder, D. J., Griffin, M. L., & Kelch, R. P. (1993). GnRH pulses—The regulators of human reproduction. *Transactions of the American Clinical and Climatological Association*, *104*, 31–46.

Martin, D., Sale, C., Cooper, S. B., & Elliott-Sale, K. J. (2018). Period prevalence and perceived side effects of hormonal contraceptive use and the menstrual cycle in elite athletes. *International Journal of Sports Physiology and Performance*, *13*(7), 926–932. https://doi.org/10.1123/ijspp.2017-0330

Martinsen, M., Bratland-Sanda, S., Eriksson, A. K., & Sundgot-Borgen, J. (2010). Dieting to win or to be thin? A study of dieting and disordered eating among adolescent elite athletes and non-athlete controls. *British Journal of Sports Medicine*, *44*(1), 70–76. https://doi.org/10.1136/bjsm.2009.068668

Matthews, C. E., Fortner, R. T., Xu, X., Hankinson, S. E., Eliassen, A. H., & Ziegler, R. G. (2012). Association between physical activity and urinary estrogens and estrogen metabolites in premenopausal women. *The Journal of Clinical Endocrinology & Metabolism*, *97*(10), 3724–3733. https://doi.org/10.1210/jc.2012-1732

McArthur, J. W., Bullen, B. A., Beitins, I. Z., Pagano, M., Badger, T. M., & Klibanski, A. (1980). Hypothalamic amenorrhea in runners of normal body composition. *Endocrine Research Communications*, *7*(1), 13–25. https://doi.org/10.3109/07435808009065956

McConnell, H., O'Connor, K., Brindle, E. L., & Williams, N. I. (2002). Validity of methods for analyzing urinary steroid data to detect ovulation in athletes. *Medicine & Science in Sports & Exercise*, *34*(11). https://journals.lww.com/acsm-msse/Fulltext/2002/11000/Validity_of_methods_for_analyzing_urinary_steroid.22.aspx

McGovern, P. G., Myers, E. R., Silva, S., Coutifaris, C., Carson, S. A., Legro, R. S., Schlaff, W. D., Carr, B. R., Steinkampf, M. P., Giudice, L. C., Leppert, P. C., & Diamond, M. P.

(2004). Absence of secretory endometrium after false-positive home urine luteinizing hormone testing. *Fertility and Sterility*, *82*(5), 1273–1277. https://doi.org/10.1016/j.fertnstert.2004.03.070

McGuigan, H. E., Hassmén, P., Rosic, N., Thornton, H. R., & Stevens, C. J. (2022). Does education improve adherence to a training monitoring program in recreational athletes? *International Journal of Sports Science & Coaching*. https://doi.org/10.1177/17479541211070789

McKay, A. K. A., Stellingwerff, T., Smith, E. S., Martin, D. T., Mujika, I., Goosey-Tolfrey, V. L., Sheppard, J., & Burke, L. M. (2022). Defining training and performance caliber: A participant classification framework. *International Journal of Sports Physiology and Performance*, *17*(2), 317–331. https://doi.org/10.1123/ijspp.2021-0451

Melin, A., Tornberg, Å. B., Skouby, S., Faber, J., Ritz, C., Sjödin, A., & Sundgot-Borgen, J. (2014). The LEAF questionnaire: A screening tool for the identification of female athletes at risk for the female athlete triad. *British Journal of Sports Medicine*, *48*(7), 540–545. https://doi.org/10.1136/bjsports-2013-093240

Melin, A., Tornberg, Å. B., Skouby, S., Møller, S. S., Faber, J., Sundgot-Borgen, J., & Sjödin, A. (2016). Low-energy density and high fiber intake are dietary concerns in female endurance athletes. *Scandinavian Journal of Medicine & Science in Sports*, *26*(9), 1060–1071. https://doi.org/10.1111/sms.12516

Meng, K., Qiu, J., Bernardot, D., Carr, A. J., Yi, L., Wang, J., & Liang, Y. (2020). The risk of low energy availability in Chinese elite and recreational female aesthetic sports athletes. *Journal of the International Society of Sports Nutrition, 17*(13). https://jissn.biomedcentral.com/articles/10.1186/s12970-020-00344-x

Mesen, T. B., & Young, S. L. (2015). Progesterone and the luteal phase. *Obstetrics and Gynecology Clinics of North America*, *42*(1), 135–151. https://doi.org/10.1016/j.ogc.2014.10.003

Metz, L., Isacco, L., & Redman, L. M. (2022). Effect of oral contraceptives on energy balance in women: A review of current knowledge and potential cellular mechanisms. *Metabolism*, *126*, 154919. https://doi.org/10.1016/j.metabol.2021.154919

Mountjoy, M., Sundgot-Borgen, J., Burke, L., Carter, S., Constantini, N., Lebrun, C., Meyer, N., Sherman, R., Steffen, K., Budgett, R., & Ljungqvist, A. (2014). The IOC consensus statement: Beyond the Female Athlete Triad—Relative Energy Deficiency in Sport (RED-S). *British Journal of Sports Medicine*, *48*(7), 491–497. https://doi.org/10.1136/bjsports-2014-093502

Murakami, H., Kawakami, R., Nakae, S., Nakata, Y., Ishikawa-Takata, K., Tanaka, S., & Miyachi, M. (2016). Accuracy of wearable devices for estimating total energy expenditure: Comparison with metabolic chamber and doubly labeled water method. *JAMA Internal Medicine*, *176*(5), 702–703. https://doi.org/10.1001/jamainternmed.2016.0152

Murakami, H., Kawakami, R., Nakae, S., Yamada, Y., Nakata, Y., Ohkawara, K., Sasai, H., Ishikawa-Takata, K., Tanaka, S., & Miyachi, M. (2019). Accuracy of 12 wearable devices for estimating physical activity energy expenditure using a metabolic chamber and the doubly labeled water method: Validation study. *JMIR MHealth and UHealth*, *7*(8), e13938. https://doi.org/10.2196/13938

Myerson, M., Gutin, B., Warren, M. P., May, M. T., Contento, I., Lee, M., Pi-Sunyer, F. X., Pierson, R. N. J., & Brooks-Gunn, J. (1991). Resting metabolic rate and energy balance

in amenorrheic and eumenorrheic runners. *Medicine & Science in Sports & Exercise*, *23*(1), 15–22.

O'Driscoll, R., Turicchi, J., Beaulieu, K., Scott, S., Matu, J., Deighton, K., Finlayson, G., & Stubbs, J. (2020). How well do activity monitors estimate energy expenditure? A systematic review and meta-analysis of the validity of current technologies. *British Journal of Sports Medicine*, *54*(6), 332–340. https://doi.org/10.1136/bjsports-2018-099643

Paludo, A. C., Paravlic, A., Dvořáková, K., & Gimunová, M. (2022). The effect of menstrual cycle on perceptual responses in athletes: A systematic review with meta-analysis. *Frontiers in Psychology*, *13*. https://www.frontiersin.org/articles/10.3389/fpsyg.2022.926854

Park, S. J., Goldsmith, L. T., Skurnick, J. H., Wojtczuk, A., & Weiss, G. (2007). Characteristics of the urinary luteinizing hormone surge in young ovulatory women. *Fertility and Sterility*, *88*(3), 684–690. https://doi.org/10.1016/j.fertnstert.2007.01.045

Pendergast, F. J., Ridgers, N. D., Worsley, A., & McNaughton, S. A. (2017). Evaluation of a smartphone food diary application using objectively measured energy expenditure. *International Journal of Behavioral Nutrition and Physical Activity*, *14*(1), 30. https://doi.org/10.1186/s12966-017-0488-9

Perry, C. D., Crane, L., Applegate, B., & Miller, P. (1996). Nutrient Intake and Psychological and Physiological Assessment in Eumenorrheic and Amenorrheic Female Athletes: A Preliminary Study. *International Journal of Sport Nutrition and Exercise Metabolism, 6*(1).

Petkus, D. L., Murray-Kolb, L. E., Scott, S. P., Southmayd, E. A., & De Souza, M. J. (2019). Iron status at opposite ends of the menstrual function spectrum. *Journal of Trace Elements in Medicine and Biology*, *51*, 169–175. https://doi.org/10.1016/j.jtemb.2018.10.016

Phumsatitpong, C., Wagenmaker, E. R., & Moenter, S. M. (2021). Neuroendocrine interactions of the stress and reproductive axes. *Frontiers in Neuroendocrinology*, *63*, 100928. https://doi.org/10.1016/j.yfrne.2021.100928

Pirke, K. M., Schweiger, U., Broocks, A., Tuschl, R. J., & Laessle, R. G. (1990). Luteinizing hormone and follicle stimulating hormone secretion patterns in female athletes with and without menstrual disturbances. *Clinical Endocrinology*, *33*(3), 345–353. https://doi.org/10.1111/j.1365-2265.1990.tb00499.x

Poehlman, EricT. (2002). Menopause, energy expenditure, and body composition. *Acta Obstetricia et Gynecologica Scandinavica*, *81*(7), 603–611. https://doi.org/10.1080/j.1600-0412.2002.810705.x

Pope, L., & Harvey-Berino, J. (2013). Burn and earn: A randomized controlled trial incentivizing exercise during fall semester for college first-year students. *Preventive Medicine*, *56*(3), 197–201. https://doi.org/10.1016/j.ypmed.2012.12.020

Prior, J. C., Naess, M., Langhammer, A., & Forsmo, S. (2015). Ovulation prevalence in women with spontaneous normal-length menstrual cycles – A population-based cohort from HUNT3, Norway. *PLOS ONE*, *10*(8), e0134473. https://doi.org/10.1371/journal.pone.0134473

Prior, J. C., Vigna, Y., Schechter, M., & Burgess, A. (1990). Spinal bone loss and ovulatory disturbances. *N Engl J Med*, *323*, 1221–1227.

Prossnitz, E. R., & Barton, M. (2011). The G-protein-coupled estrogen receptor GPER in health and disease. *Nature Reviews Endocrinology*, *7*(12), 715–726. https://doi.org/10.1038/nrendo.2011.122

Ravi, S., Waller, B., Valtonen, M., Villberg, J., Vasankari, T., Parkkari, J., Heinonen, O. J., Alanko, L., Savonen, K., Vanhala, M., Selänne, H., Kokko, S., & Kujala, U. M. (2021). Menstrual dysfunction and body weight dissatisfaction among Finnish young athletes and non-athletes. *Scandinavian Journal of Medicine & Science in Sports*, *31*(2), 405–417. https://doi.org/10.1111/sms.13838

Reed, J. L., Bowell, J. L., Hill, B. R., Williams, B. A., De Souza, M. J., & Williams, N. I. (2011). Exercising women with menstrual disturbances consume low energy dense foods and beverages. *Appl Physiol Nutr Metab*, *36*(3), 382–394. https://doi.org/10.1139/h11-030

Reed, J. L., De Souza, M. J., Mallinson, R. J., Scheid, J. L., & Williams, N. I. (2015). Energy availability discriminates clinical menstrual status in exercising women. *Journal of the International Society of Sports Nutrition*, *12*(1), 11. https://doi.org/10.1186/s12970-015-0072-0

Riaz, Y., & Parekh, U. (2022). Oligomenorrhea. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK560575/

Rocha-Rodrigues, S., Sousa, M., Lourenço Reis, P., Leão, C., Cardoso-Marinho, B., Massada, M., & Afonso, J. (2021). Bidirectional interactions between the menstrual cycle, exercise training, and macronutrient intake in women: A Review. *Nutrients*, *13*(2), Article 2. https://doi.org/10.3390/nu13020438

Rock, C. L., Gorenflo, D. W., Drewnowski, A., & Demitrack, M. A. (1996). Nutritional characteristics, eating pathology, and hormonal status in young women. *The American Journal of Clinical Nutrition*, *64*(4), 566–571. https://doi.org/10.1093/ajcn/64.4.566

Rogers, M. A., Drew, M. K., Appaneal, R., Lovell, G., Lundy, B., Hughes, D., Vlahovich, N., Waddington, G., & Burke, L. M. (2021). The utility of the low energy availability in females questionnaire to detect markers consistent with low energy availability-related conditions in a mixed-sport cohort. *International Journal of Sport Nutrition and Exercise Metabolism*, *31*(5), 427–437. https://doi.org/10.1123/ijsnem.2020-0233

Rosetta, L., Condé da Silva Fraga, E., & Mascie-Taylor, C. G. (2001). Relationship between self-reported food and fluid intake and menstrual disturbance in female recreational runners. *Annals of Human Biology*, *28*(4), 444–454. https://doi.org/10.1080/03014460010019759

Rosetta, L., Harrison, G. A., & Read, G. F. (1998). Ovarian impairments of female recreational distance runners during a season of training. *Annals of Human Biology*, *25*(4), 345–357. https://doi.org/10.1080/03014469800005692

Rosetta, L., Williams, C., Brooke-Wavell, K. S. F., & Norgan, N. G. (1998). Diet and body composition of female recreational runners of differing menstrual status. *Journal of Sports Sciences*, *16*(7), 629–637. https://doi.org/10.1080/026404198366434

Roupas, N., & Georgopoulos, N. (2011). Menstrual function in sports. *HORMONES*, *10*(2), 104–116. https://doi.org/10.14310/horm.2002.1300

Saei Ghare Naz, M., Rostami Dovom, M., & Ramezani Tehrani, F. (2020). The menstrual disturbances in endocrine disorders: A narrative review. *International Journal of Endocrinology and Metabolism*, *18*(4), e106694. https://doi.org/10.5812/ijem.106694

Saketos, M., Sharma, N., & Santoro, N. F. (1993). Suppression of the hypothalamic-pituitary-ovarian axis in normal women by glucocorticoids. *Biology of Reproduction*, *49*(6), 1270–1276. https://doi.org/10.1095/biolreprod49.6.1270

Sanders, D., Abt, G., Hesselink, M. K., Myers, T., & Akubat, I. (2017). Methods of monitoring training load and their relationships to changes in fitness and performance in competitive road cyclists. *International Journal of Sports Physiology and Performance*, *12*(5).

Sanders, D., Van Erp, T., & De Koning, J. J. (2019). Intensity and load characteristics of professional road cycling: Differences between men's and women's races. *International Journal of Sports Physiology and Performance*, *14*(3), 296–302. https://doi.org/10.1123/ijspp.2018-0190

Santaro, N., Crawford, S., Allsworth, J., Gold, E., Greendale, A., Korenman, S., Lasley, B. L., McConnell, D., McGaffigan, P., Midgely, R., Shocken, M., Sowers, M., & Weiss, G. (2002). Assessing menstrual cycles with urinary hormone assays. *Am J Physiol Endocrinol Metab, 284*. https://doi.org/10.1152/ajpendo.00381.2002

Schaal, K., VanLoan, M. D., Hausswirth, C., & Casazza, G. A. (2021). Decreased energy availability during training overload is associated with non-functional overreaching and suppressed ovarian function in female runners. *Applied Physiology, Nutrition, and Metabolism*, *46*(10), 1179–1188. https://doi.org/10.1139/apnm-2020-0880

Schaumberg, M. A., Jenkins, D. G., Janse de Jonge, X. A. K., Emmerton, L. M., & Skinner, T. L. (2017). Three-step method for menstrual and oral contraceptive cycle verification. *Journal of Science and Medicine in Sport*, *20*(11), 965–969. https://doi.org/10.1016/j.jsams.2016.08.013

Schliep, K. C., Mumford, S. L., Hammoud, A. O., Stanford, J. B., Kissell, K. A., Sjaarda, L. A., Perkins, N. J., Ahrens, K. A., Wactawski-Wende, J., Mendola, P., & Schisterman, E. F. (2014). Luteal phase deficiency in regularly menstruating women: Prevalence and overlap in identification based on clinical and biochemical diagnostic criteria. *The Journal of Clinical Endocrinology & Metabolism*, *99*(6).

Schliep, K. C., Schisterman, E. F., Mumford, S. L., Pollack, A. Z., Perkins, N. J., Ye, A., Zhang, C. J., Stanford, J. B., Porucznik, C. A., Hammoud, A. O., & Wactawski-Wende, J. (2013). Energy-containing beverages: Reproductive hormones and ovarian function in the BioCycle Study. *The American Journal of Clinical Nutrition*, *97*(3), 621–630. https://doi.org/10.3945/ajcn.111.024752

Schliep, K. C., Schisterman, E. F., Mumford, S. L., Pollack, A. Z., Zhang, C., Ye, A., Stanford, J. B., Hammoud, A. O., Porucznik, C. A., & Wactawski-Wende, J. (2012). Caffeinated beverage intake and reproductive hormones among premenopausal women in the BioCycle Study. *The American Journal of Clinical Nutrition*, *95*(2), 488–497. https://doi.org/10.3945/ajcn.111.021287

Schwartz, B., Cumming, D. C., Riordan, E., Selye, M., Yen, S. S. C., & Rebar, R. W. (1981). Exercise-associated amenorrhea: A distinct entity? *American Journal of Obstetrics and Gynecology*, *141*(5), 662–670. https://doi.org/10.1016/S0002-9378(15)33308-1

Seppä, S., Kuiri-Hänninen, T., Holopainen, E., & Voutilainen, R. (2021). Management of endocrine disease: Diagnosis and management of primary amenorrhea and female delayed puberty. *European Journal of Endocrinology*, *184*(6), R225–R242. https://doi.org/10.1530/EJE-20-1487

Shangold, M. M., & Levine, H. S. (1982). The effect of marathon training upon menstrual function. *American Journal of Obstetrics and Gynecology*, *143*(8), 862–869. https://doi.org/10.1016/0002-9378(82)90464-1

Sharps, F. R. J., Wilson, L. J., Graham, C. A.-M., & Curtis, C. (2021). Prevalence of disordered eating, eating disorders and risk of low energy availability in professional, competitive and recreational female athletes based in the United Kingdom. *European Journal of Sport Science*, *0*(0), 1–7. https://doi.org/10.1080/17461391.2021.1943712

Shirtcliff, E. A., Granger, D. A., Schwartz, E. B., Curran, M. J., Booth, A., & Overman, W. H. (2000). Assessing estradiol in biobehavioral studies using saliva and blood spots: Simple radioimmunoassay protocols, reliability, and comparative validity. *Hormones and Behavior*, *38*(2), 137–147. https://doi.org/10.1006/hbeh.2000.1614

Shultz, S. J., Wideman, L., Montgomery, M. M., & Levine, B. J. (2011). Some sex hormone profiles are consistent over time in normal menstruating women: Implications for sports injury epidemiology. *British Journal of Sports Medicine*, *45*(9), 735–742. https://doi.org/10.1136/bjsm.2009.064931

Silberstein, S. D., & Merriam, G. R. (2000). Physiology of the menstrual cycle. *Cephalalgia*, *20*(3), 148–154.

Sim, A., & Burns, S. F. (2021). Review: Questionnaires as measures for low energy availability (LEA) and relative energy deficiency in sport (RED-S) in athletes. *Journal of Eating Disorders*, *9*(1), 41. https://doi.org/10.1186/s40337-021-00396-7

Sitruk-Ware, R., & El-Etr, M. (2013). Progesterone and related progestins: Potential new health benefits. *Climacteric*, *16*(sup1), 69–78. https://doi.org/10.3109/13697137.2013.802556

Skinner, M. (2018). *Encyclopedia of Reproduction* (2nd ed.).

Slater, J., McLay-Cooke, R., Brown, R., & Black, K. (2016). Female recreational exercisers at risk for low energy availability. *International Journal of Sport Nutrition and Exercise Metabolism*, *26*(5), 421–427. https://doi.org/10.1123/ijsnem.2015-0245

Smith, E. S., McKay, A. K. A., Ackerman, K. E., Harris, R., Elliott-Sale, K. J., Stellingwerff, T., & Burke, L. M. (2022). Methodology review: A protocol to audit the representation of female athletes in sports science and sports medicine research. *International Journal of Sport Nutrition and Exercise Metabolism*, *32*(2), 114–127. https://doi.org/10.1123/ijsnem.2021-0257

Sonntag, B., & Ludwig, M. (2012). An integrated view on the luteal phase: Diagnosis and treatment in subfertility. *Clin Endocrinol, 77*(4), 500–507.

Soules, M. R., Mclachan, R. I., EK, M., Dahl, K. D., Cohen, N. L., & Bremner, W. J. (1989). Luteal phase deficiency: Characterization of reproductive hormones over the menstrual cycle. *The Journal of Clinical Endocrinology & Metabolism*, *69*(4), 804–812. https://doi.org/10.1210/jcem-69-4-804

Soumpasis, I., Grace, B., & Johnson, S. (2020). Real-life insights on menstrual cycles and ovulation using big data*. Human Reproduction Open, 2020*(2), 1–9.

Stein, D. G., & Wright, D. W. (2010). Progesterone in the clinical treatment of acute traumatic brain injury. *Expert Opinion on Investigational Drugs*, *19*(7), 847–857. https://doi.org/10.1517/13543784.2010.489549

Stellingwerff, T., Heikura, I. A., Meeusen, R., Bermon, S., Seiler, S., Mountjoy, M., & Burke, L. (2021). Overtraining syndrome (OTS) and Relative Energy Deficiency in Sport (RED-S): Shared pathways, symptoms and complexities. *Sports Medicine*. https://doi.org/10.1007/s40279-021-01491-0

Stoddard, J. L., Dent, C. W., Shames, L., & Bernstein, L. (2006). Exercise training effects on premenstrual distress and ovarian steroid hormones. *European Journal of Applied Physiology*, *99*(1), 27–37. https://doi.org/10.1007/s00421-006-0313-7

Strock, N. C. A., Koltun, K. J., Mallinson, R. J., Williams, N. I., & De Souza, M. J. (2020). Characterizing the resting metabolic rate ratio in ovulatory exercising women over 12 months. *Scandinavian Journal of Medicine & Science in Sports*, *30*(8), 1337–1347. https://doi.org/10.1111/sms.13688

Strock, N. C. A., Koltun, K. J., Southmayd, E. A., Williams, N. I., & Souza, M. J. D. (2020). Indices of resting metabolic rate accurately reflect energy deficiency in exercising

women. *International Journal of Sport Nutrition and Exercise Metabolism*, *30*(1), 14–24. https://doi.org/10.1123/ijsnem.2019-0199

Suh, B. Y., & Betz, G. (1993). Altered luteinizing hormone pulse frequency in early follicular phase of the menstrual cycle with luteal phase defect patients in women. *Fertility and Sterility*, *60*(5), 800–805. https://doi.org/10.1016/S0015-0282(16)56279-7

Teixeira, V., Voci, S. M., Mendes-Netto, R. S., & da Silva, D. G. (2018). The relative validity of a food record using the smartphone application MyFitnessPal. *Nutrition & Dietetics*, *75*(2), 219–225. https://doi.org/10.1111/1747-0080.12401

Thompson, S. H., & Gabriel, M. (2004). Risk factors for the female athlete triad among female collegiate and noncollegiate athletes. *Physical Educator*, *61*(4), 200–212.

Thong, F., McLean, C., & Graham, T. (2007). Plasma leptin in female athletes: Relationship with body fat, reproductive, nutritional, and endocrine factors. *J Appl Physiol*, *88*. https://doi.org/10.1152/jappl.2000.88.6.2037

Toth, M. J., Poehlman, E. T., Matthews, D. E., Tchernof, A., & MacCoss, M. J. (2001). Effects of estradiol and progesterone on body composition, protein synthesis, and lipoprotein lipase in rats. *American Journal of Physiology-Endocrinology and Metabolism*, *280*(3), E496–E501. https://doi.org/10.1152/ajpendo.2001.280.3.E496

TrainingPeaks. (n.d.). Retrieved July 4, 2022, from https://www.trainingpeaks.com/

Vaiksaar, S., Jürimäe, J., Mäestu, J., Purge, P., Kalytka, S., Shakhlina, L., & Jürimäe, T. (2011). No effect of menstrual cycle phase on fuel oxidation during exercise in rowers. *European Journal of Applied Physiology*, *111*(6), 1027–1034. https://doi.org/10.1007/s00421-010-1730-1

Veldhuis, J. D., Evans, W. S., Demers, L. M., Thorner, M. O., Wakat, D., & Rogol, A. D. (1985). Altered Neuroendocrine Regulation of Gonadotropin Secretion in Women Distance

Runners. *The Journal of Clinical Endocrinology & Metabolism*, *61*(3), 557–563. https://doi.org/10.1210/jcem-61-3-557

Versini, A., Ramoz, N., Le Strat, Y., Scherag, S., Ehrlich, S., Boni, C., Hinney, A., Hebebrand, J., Guelfi, J.-D., & Gorwood, P. (2010). Estrogen Receptor 1 Gene (ESR1) is associated with restrictive anorexia nervosa. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *35*, 1818–1825. https://doi.org/10.1038/npp.2010.49

Viner, R. T., Harris, M., Berning, J. R., & Meyer, N. L. (2015). Energy availability and dietary patterns of adult male and female competitive cyclists with lower than expected bone mineral density. *International Journal of Sport Nutrition and Exercise Metabolism*, *25*(6), 594–602. https://doi.org/10.1123/ijsnem.2015-0073

Wallach, E. E., Shoham, Z., Schachter, M., Loumaye, E., Weissman, A., MacNamee, M., & Insler, V. (1995). The luteinizing hormone surge—the final stage in ovulation induction: Modern aspects of ovulation triggering. *Fertility and Sterility*, *64*(2), 237–251. https://doi.org/10.1016/S0015-0282(16)57717-6

Warren, M. P., & Perlroth, N. E. (2001). The effects of intense exercise on the female reproductive system. *Journal of Endocrinology*, *170*(1), 3–11. https://doi.org/10.1677/joe.0.1700003

Wicks, J. R., & Oldridge, N. B. (2016). How accurate is the prediction of maximal oxygen uptake with treadmill testing? *PLOS ONE*, *11*(11), e0166608. https://doi.org/10.1371/journal.pone.0166608

Wicks, J. R., Oldridge, N. B., Nielsen, L. K., & Vickers, C. E. (2011). HR Index-A simple method for the prediction of oxygen uptake. *Medicine & Science in Sports & Exercise*, *43*(10), 2005. https://doi.org/10.1249/MSS.0b013e318217276e

Wideman, L., Montgomery, M. M., Levine, B. J., Beynnon, B. D., & Shultz, S. J. (2013). Accuracy of calendar-based methods for assigning menstrual cycle phase in women. *Sports Health*, *5*(2), 143–149.

Williams, N. I., Bullen, B. A., McARTHUR, J. W., Skrinar, G. S., & Turnbull, B. A. (1999). Effects of short-term strenuous endurance exercise upon corpus luteum function. *Medicine & Science in Sports & Exercise*, *31*(7), 949–958.

Williams, N. I., Helmreich, D. L., Parfitt, D. B., Caston-Balderrama, A., & Cameron, J. L. (2001). Evidence for a causal role of low energy availability in the induction of menstrual cycle disturbances during strenuous exercise training. *The Journal of Clinical Endocrinology and Metabolism*, *86*(11), 5184–5193. https://doi.org/10.1210/jcem.86.11.8024

Williams, N. I., Leidy, H. J., Hill, B. R., Lieberman, J. L., Legro, R. S., & Souza, M. J. D. (2015). Magnitude of daily energy deficit predicts frequency but not severity of menstrual disturbances associated with exercise and caloric restriction. *American Journal of Physiology-Endocrinology and Metabolism*, *308*(1), E29–E39. https://doi.org/10.1152/ajpendo.00386.2013

Witkoś, J., & Hartman-Petrycka, M. (2022). The Female Athlete Triad-the impact of running and type of diet on the regularity of the menstrual cycle assessed for recreational runners. *PeerJ*, *10*, e12903. https://doi.org/10.7717/peerj.12903

Woods, A. L., Rice, A. J., Garvican-Lewis, L. A., Wallett, A. M., Lundy, B., Rogers, M. A., Welvaert, M., Halson, S., McKune, A., & Thompson, K. G. (2018). The effects of intensified training on resting metabolic rate (RMR), body composition and performance in trained cyclists. *PLOS ONE*, *13*(2), e0191644. https://doi.org/10.1371/journal.pone.0191644

Worthman, C. M., Stallings, J. F., & Hofman, L. F. (1990). Sensitive salivary estradiol assay for monitoring ovarian function. *Clinical Chemistry*, *36*(10), 1769–1773. https://doi.org/10.1093/clinchem/36.10.1769

Xu, Y., Nedungadi, T. P., Zhu, L., Sobhani, N., Irani, B. G., Davis, K. E., Zhang, X., Zou, F., Gent, L. M., Hahner, L. D., Khan, S. A., Elias, C. F., Elmquist, J. K., & Clegg, D. J. (2011). Distinct hypothalamic neurons mediate estrogenic effects on energy homeostasis and reproduction. *Cell Metabolism*, *14*(4), 453–465. https://doi.org/10.1016/j.cmet.2011.08.009

Yen, S. S. C. (1979). Neuroendocrine regulation of the menstrual cycle. *Hospital Practice*, *14*(3), 83–97. https://doi.org/10.1080/21548331.1979.11707503

Yesildere Saglam, H., & Orsal, O. (2020). Effect of exercise on premenstrual symptoms: A systematic review. *Complementary Therapies in Medicine*, *48*, 102272. https://doi.org/10.1016/j.ctim.2019.102272

Yu, K., He, Y., Hyseni, I., Pei, Z., Yang, Y., Xu, P., Cai, X., Liu, H., Qu, N., Liu, H., He, Y., Yu, M., Liang, C., Yang, T., Wang, J., Gourdy, P., Arnal, J.-F., Lenfant, F., Xu, Y., & Wang, C. (2020). 17β-estradiol promotes acute refeeding in hungry mice via membrane-initiated ERα signaling. *Molecular Metabolism*, *42*, 101053. https://doi.org/10.1016/j.molmet.2020.101053

Zavala, E., Voliotis, M., Zerenner, T., Tabak, J., Walker, J. J., Li, X. F., Terry, J. R., Lightman, S. L., O'Byrne, K., & Tsaneva-Atanasova, K. (2020). Dynamic hormone control of stress and fertility. *Frontiers in Physiology*, *11*. https://www.frontiersin.org/articles/10.3389/fphys.2020.598845

Ziomkiewicz, A., Ellison, P. T., Lipson, S. F., Thune, I., & Jasienska, G. (2008). Body fat, energy balance and estradiol levels: A study based on hormonal profiles from complete

menstrual cycles. *Human Reproduction*, *23*(11), 2555–2563.

https://doi.org/10.1093/humrep/den213

APPENDIX A: SCREENING FORM

Please enter the information asked below.

- ○ Name _____
- ○ Phone _____
- ○ Email _____
- ○ Age _____

Do you exercise an average of 5 days per week for a minimum of 30 minutes each time?

- ○ Yes
- ○ No

Have you used any hormonal contraceptive (e.g., birth control pill, IUD) in the last three months?

- ○ Yes
- ○ No

Do you have a menstrual cycle every 21-45 days?

- ○ Yes
- ○ Maybe
- ○ No

Are you pregnant or trying to get pregnant?

- ○ Yes
- ○ Maybe
- ○ No

Are you currently dieting or trying to lose weight?

- ○ Yes
- ○ No

Do you have any metal that is not removable? Examples are but not limited to hip/knee replacements, spinal rods, and piercings not able to be removed.

- ○ Yes
- ○ No

X→

Do you smoke?

- ○ Yes
- ○ No

X→

Do you use any medications?

- ○ Yes
- ○ No

Please list your medications.

_____

_____

_____

_____

_____

Do you currently take any supplements? Please check all that apply.

- [ ] Creatine
- [ ] Protein
- [ ] Multi-vitamins
- [ ] Vitamin D
- [ ] Iron supplementation
- [ ] Probiotics
- [ ] Caffeine
- [ ] Fish Oil
- [ ] Beta Alanine
- [ ] I don't take any supplements
- [ ] Other. Please list. _____

Have you ever been diagnosed with any of the following? Please check all that apply.

- [ ] Eating disorder (Anorexia Nervosa, Bulima)
- [ ] Disordered Eating (irregular eating habits but not severe enough to be diagnosed with an eating disorder)
- [ ] Stress Fractures
- [ ] Low bone density/Osteopenia/Osteoporosis
- [ ] Hypothyroidism
- [ ] Oligomenorrhea (reduced periods)
- [ ] Amenorrhea (no periods)
- [ ] Endometriosis
- [ ] Polycystic Ovarian Syndrome
- [ ] I have never been diagnosed with any items listed
- [ ] Any other gynecological or pituitary diagnosis not listed here: _____

Have you had, or do you presently have any of the following? Please check all that apply.

- [ ] Heart disease
- [ ] Diabetes/Prediabetes
- [ ] High blood pressure
- [ ] Low blood pressure
- [ ] Cancer
- [ ] Seizures
- [ ] Recent operation
- [ ] Muscle or joint problems
- [ ] Kidney disease
- [ ] Lung disease
- [ ] Any other diagnosis not listed here: _____

I have never been diagnosed with any items listed

APPENDIX B: LOW ENERGY AVAILABILITY IN FEMALES QUESTIONNAIRE (LEAF-Q)

How many days absence from training or participation in competition due to injuries have you had in the last year?

- ○ 1-7 days
- ○ 8-14 days
- ○ 15-21 days
- ○ 22 days or more

What kind of injuries have you had in the last year?

- [ ] Muscular Strain/Tear
- [ ] Stress fractures
- [ ] Iliotibial (IT) band syndrome
- [ ] Knee injury
- [ ] Lower back injury
- [ ] Hamstring injury
- [ ] Ankle injury
- [ ] Foot injury/plantar fasciitis
- [ ] I haven't had any injuries in the last year
- [ ] Other: please list _____

Do you feel gaseous or bloated in the abdomen when you do not have your period?

- ○ Yes, several times a day
- ○ Yes, several times a week
- ○ Yes, once or twice a week or more seldom
- ○ Rarely or never

Do you get cramps or stomach ache which cannot be related to your menstruation?

- ○ Yes, several times a day
- ○ Yes, several times a week
- ○ Yes, once or twice a week or more seldom
- ○ Rarely or never

How often do you have bowel movements on average?

- Several times a day
- Once a day
- Every second day
- Twice a week
- Once a week or more rarely

How would you describe your normal stool?

- Normal (soft)
- Diarrhea-like (watery)
- Hard and dry

Do you use oral contraceptives?

- ○ Yes
- ○ No

Why do you use oral contraceptives?

- ○ Contraception
- ○ Reduction of menstruation pains
- ○ Reduction of bleeding
- ○ To regulate the menstrual cycle in relation to performances etc..
- ○ Otherwise menstruation stops
- ○ Other _____

Have you used oral contraceptives previously?

- ○ Yes
- ○ No

How long ago did you use oral contraceptives?

- ○ 0-6 months
- ○ 6-12 months
- ○ More than 12 months

For how long?

- ○ 0-6 months
- ○ 6-12 months
- ○ More than 12 months

Do you use any other kind of hormonal contraceptives? (e.g. hormonal implant or coil [IUD])

- ○ Yes
- ○ No

What kind of contraceptive?

- ○ Hormonal patches
- ○ Hormonal ring
- ○ Hormonal coil/intrauterine device (IUD)
- ○ Hormonal implant
- ○ Depo-Provera Injection
- ○ Other

How old were when you had your first period?

- ○ 11 years or younger
- ○ 12-14 years
- ○ 15 years or older
- ○ I don’t remember
- ○ I have never menstruated

Did your first menstruation come naturally (by itself)?

- ○ Yes
- ○ No
- ○ I don't remember

What kind of treatment was used to start your menstrual cycle?

- ○ Hormonal treatment
- ○ Weight gain
- ○ Reduced amount of exercise
- ○ Other

X→

Do you have normal menstruation?

- ○ Yes
- ○ No
- ○ I don't know

X→

When was your last period?

- 0-4 weeks ago
- 1-2 months ago
- 3-4 months ago
- 5 months ago or more

X→

Are your periods regular? (Every 28th to 34th day)

- Yes, most of the time
- No, mostly not

X→

For how many days do you normally bleed?

- ○ 1-2 days
- ○ 3-4 days
- ○ 5-6 days
- ○ 7-8 days
- ○ 9 days or more

Have you ever had problems with heavy menstrual bleeding?

- ○ Yes
- ○ No

How many periods have you had during the last year?

- 12 or more
- 9-11
- 6-8
- 3-5
- 0-2

When did you have your last period?

- 2-3 months ago
- 4-5 months ago
- 6 months ago or more
- I’m pregnant and therefore do not menstruate
- My hormonal contraceptive or medical reasons stopped my menstruation

Have your periods ever stopped for 3 consecutive months or longer (besides pregnancy or because of medication)?

- ○ No, never
- ○ Yes, it has happened before
- ○ Yes, that’s the situation now

X→

Do you experience that your menstruation changes when you increase your exercise intensity, frequency, or duration?

- ○ Yes
- ○ No

X→

How? (Check one or more options)

- [ ] I bleed less
- [ ] I bleed fewer days
- [ ] My menstruation stops
- [ ] I bleed more
- [ ] I bleed more days

End of Block: LEAF-Q

APPENDIX C: ACUTE RECOVERY AND STRESS SCALE

ARSS Below there is a list of expressions that describe different states of recovery and stress. Please rate each item and mark the number that most closely applies to you **right now**.

	Does not apply at all 0	1	2	3	4	5	Fully applies 6
recovered	○	○	○	○	○	○	○
muscle exhaustion	○	○	○	○	○	○	○
pleased	○	○	○	○	○	○	○
unmotivated	○	○	○	○	○	○	○
attentive	○	○	○	○	○	○	○
feeling down	○	○	○	○	○	○	○
strong	○	○	○	○	○	○	○
tired	○	○	○	○	○	○	○

rested	○	○	○	○	○	○	○
muscle fatigue	○	○	○	○	○	○	○
stable	○	○	○	○	○	○	○
sluggish	○	○	○	○	○	○	○
receptive	○	○	○	○	○	○	○
stressed	○	○	○	○	○	○	○
physically capable	○	○	○	○	○	○	○
worn-out	○	○	○	○	○	○	○
muscle relaxation	○	○	○	○	○	○	○
unenthusiastic	○	○	○	○	○	○	○
in a good mood	○	○	○	○	○	○	○

annoyed	○	○	○	○	○	○	○
mentally alert	○	○	○	○	○	○	○
muscle soreness	○	○	○	○	○	○	○
energetic	○	○	○	○	○	○	○
overloaded	○	○	○	○	○	○	○
physically relaxed	○	○	○	○	○	○	○
muscle stiffness	○	○	○	○	○	○	○
having everything under control	○	○	○	○	○	○	○
lacking energy	○	○	○	○	○	○	○
concentrated	○	○	○	○	○	○	○
short-tempered	○	○	○	○	○	○	○

full of power	○	○	○	○	○	○	○
physically exhausted	○	○	○	○	○	○	○