THE ROLE OF LOW ENERGY AVAILABILITY IN PREDICTING AN ENERGY DEFICIENCY AND MENSTRUAL DISTURBANCES IN RECREATIONAL AND COMPETITIVE FEMALE ATHLETES

A Dissertation in
Kinesiology
by
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Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

May 2012
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ABSTRACT

The Female Athlete Triad is a syndrome of three interrelated conditions: amenorrhea, osteoporosis, and low energy availability (EA). These conditions, either alone or in combination, pose significant health risks to exercising women. The overall goal of this dissertation was to help increase our understanding of EA, defined as the difference between dietary energy intake and exercise energy expenditure, normalized to kilograms of lean body mass, as a tool for assessing energy and menstrual status in a field setting. Short-term laboratory studies in sedentary women have identified a threshold of EA below which reproductive hormone secretion is disrupted, i.e., < 30 kcal/kg lean body mass but no studies have thoroughly examined the association of low EA and menstrual disturbances in trained women using more conventional methods to assess EA. We therefore assessed the prevalence of low EA (< 30 kcal/kg lean body mass) and its association with menstrual status and other indices of energy balance in exercising women outside controlled laboratory conditions.

Study 1 was designed to assess the risk of low EA in Division I female soccer players during the pre, mid, and post season. We demonstrated that although the mean EA of the group was not low, a concerning percentage of these athletes exhibited low EA at some point during the season and that negative eating attitudes were also observed in athletes with low EA. Study 2 was designed to test whether EA discriminates disruptions in menstrual function in exercising premenopausal women. In contrast to our hypothesis, we demonstrated that EA did not discriminate menstrual status in a large sample of exercising women when using conventional methods to assess EA including self-reported diet logs, exercise logs, and heart rate monitors. The purpose of Study 3 was to determine
if exercising women with menstrual disturbances who display reproductive recovery experience a greater increase in EA when compared to exercising women with menstrual disturbances who do not display reproductive recovery. We also tested the association between crossing a threshold value of 30 kcal/kg lean body mass and reproductive recovery. In contrast to our hypothesis, we demonstrated that trained women with menstrual disturbances who displayed reproductive recovery, whether defined as the resumption of menstrual bleeding or the resumption of menstrual bleeding preceded by ovulation, did not experience a greater increase in EA when compared to their counterparts who did not display reproductive recovery. Study 4 was designed to assess which of several indices of energy status best predicts the presence of an energy deficit and the presence of menstrual disturbances. We demonstrated that EA best predicts an energy deficiency, but that the ratio of measured to predicted resting energy expenditure best predicts a menstrual disturbance in exercising premenopausal women.

Taken together, these data suggest that EA may not be a useful tool in assessing menstrual status in exercising women. Since an energy deficit is known to be related to disruptions in the menstrual cycle, the strong correlation between EA and energy deficiency we observed in Study 4, in the face of a non-significant association between EA and menstrual status found in Studies 2 and 3, is paradoxical. Possible explanations for the lack of association between EA and menstrual status include high variability in the measurement of the components of the EA index, dependence of menstrual status on aspects of energy balance not captured by EA, low statistical power, under or over reporting of dietary energy intake, and/or misclassification of menstrual status. Future studies are needed to more clearly understand the specific associations between the
components of energy balance and menstrual status and to improve the accuracy of EA measurements.
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Acknowledgements

This dissertation would not have been possible without the support and guidance of my mentor, Dr. Nancy Williams. Thank you for understanding and supporting my passion to work with elite athletes. It is because of you that I have strengthened my ability to think independently and critically evaluate every step of the scientific process. It was a privilege working with you.

I wish to thank Dr. Mary Jane De Souza for giving me the opportunity to pursue my doctoral studies at the Pennsylvania State University. The opportunities you afforded me with were greatly appreciated. I also wish to thank you for all of your valuable insights throughout the data analysis and writing process.

I would like to thank Dr. Cynthia Bartok and Dr. Terryl Hartman for serving on my dissertation committee. I am grateful for your feedback and suggestions in helping to improve this dissertation. It was truly a pleasure working with you.

I am grateful to my colleagues in the Women’s Health and Exercise Lab, the nurses and staff of the General Clinical Research Center, the Penn State Division I women’s soccer team, and the Department of Kinesiology for all of your assistance, support, and encouragement throughout my doctoral studies and in helping to make this dissertation possible.

Finally, I would like to thank my family, friends, and most importantly, my husband, Fraser Reed, for his unconditional love, support, and patience throughout my graduate studies. This dissertation would not have been possible without you.
Chapter 1

Introduction

The Female Athlete Triad is a syndrome of three interrelated conditions: amenorrhea, osteoporosis, and low energy availability (EA). These conditions, either alone or in combination, pose significant health risks to exercising women (171). Findings from several studies manipulating dietary energy intake (EI) and exercise energy expenditure (EEE) (32, 193, 256-257, 259-260) or EI alone (137, 178, 188-190, 210) support the role of low EA in the development of menstrual disturbances in exercising women. Studies in mammalian species have shown a causal role for low EA in the induction of reproductive disturbances (246, 257-258).

With respect to investigations on the impact of exercise on reproductive function, EA has been operationally defined by one investigator (144) as EI minus EEE relative to kilograms of lean body mass (LBM) i.e., (EA = EI-EEE/kg LBM) represents the amount of EI remaining after exercise training for all other metabolic processes such as reproduction, thermoregulation, cellular maintenance, and locomotion (134, 246). Short term studies manipulating EI and EEE have shown that negative metabolic, reproductive, and bone related changes occur when EA drops below 30 kcal/kg LBM (103, 144) but these studies have been performed under strict laboratory controlled conditions, have only been performed in previously sedentary women, and have not examined how the menstrual cycle is impacted by certain levels of EA.
In real life, exercising women who restrict EI, exercise for long periods of time, and limit their food choices are at the greatest risk for low EA (<30 kcal/kg LBM) (171). No studies to date have examined the prevalence of low EA (<30 kcal/kg LBM) or how the risk of low EA changes across a season in female athletes who have been shown to consume low EI (40) relative to high EEE (213). This is important because an unexpected high prevalence of eating disorders (28%) has been observed among female athletes participating in ball-game sports (224) and disordered eating attitudes might predispose female athletes to consume lower EI (84, 196). Eating behaviors such as low EI of particular meals and number of eating occasions have also been shown to impact total EI (92, 233). No studies to date have examined the association between eating attitudes, behaviors, and low EA. To address these important and practical questions, we conducted a prospective study in Division I female soccer players to assess the risk of low EA (<30 kcal/kg LBM) during the pre, mid, and post season. We also examined whether negative eating attitudes, lower EI at particular meals, and number of eating occasions might be associated with lower EI and thus low EA in Division I female soccer players. From this first study, we hoped to gain an understanding of the risk and prevalence of low EA (<30 kcal/kg LBM) across a season in Division I female soccer players. We also hoped to gain an understanding of the eating attitudes and eating behaviors that contribute to low EA.

Prospective exercise training studies suggest that low EA is causally related to reproductive disturbances in exercising women (32, 256-257, 260). Reductions in luteinizing hormone (LH) pulsatility have been observed below an EA of 30 kcal/kg LBM in previously sedentary women during short-term manipulations of EI and EEE in a
controlled laboratory setting (144). Although these studies (97, 103, 136, 138, 144) define a critical EA threshold of 30 kcal/kg LBM) for the initiation of reduced LH pulsatility, few investigators have examined whether an EA of 30 kcal/kg LBM discriminates normal ovulatory menstrual function from either subtle (luteal phase defects or anovulation) or severe (oligomenorrhea or amenorrhea) menstrual disturbances in exercising women. The most severe reductions in LH pulsatility have been observed in amenorrheic (142, 240) runners. Less severe reductions in LH pulsatility have been observed in exercising women with eumenorrhea (50), anovulatory menstrual cycles (187), and oligomenorrhea (240). In addition, no studies have examined whether an EA of 30 kcal/kg LBM or lower is associated with a dose response relationship with menstrual disturbances of increasing severity progressing from luteal phase defects and anovulatory menstrual cycles to oligomenorrhea, and amenorrhea (68).

To address these above mentioned gaps in the literature, we conducted a cross sectional study in trained premenopausal exercising women to assess the role of a 30 kcal/kg LBM threshold in differentiating menstrual status and whether a dose response relationship exists such that menstrual disturbances increase in severity below and EA of 30 kcal/kg LBM (68). From this second study, we hoped to gain an understanding of the role of EA as a variable in discriminating menstrual status in free living trained exercising premenopausal women. An important benefit of knowing this association would be that EA might be an easily measured proxy indicator of the risk for further Female Athlete Triad related health consequences.

While it is important to understand the role of EA as a variable in the induction of menstrual disturbances, it is equally important to understand its role in the reversal of
reproductive dysfunction. Studies in animals (258) and humans (83, 86, 163, 258) have demonstrated the effectiveness of either improved EI (83, 86, 163, 258), decreased EEE (249), or the manipulation of both (73, 115) to improve indices of reproductive function. Although the physiological relevance of EA in reproductive function has been shown, an important practical question that has not been adequately explored is the magnitude of EA, defined as EI minus EEE relative to kilograms of LBM, that is needed to reverse menstrual disturbances in exercising women. In addition, the relevance of a 30 kcal/kg LBM threshold in the reversal of menstrual disturbances has not been examined. To address these important questions, we conducted a randomized controlled trial in trained exercising premenopausal women with exercise associated menstrual disturbances to examine whether exercising women who recover reproductive function experience a greater change in EA than exercising women who do not recover reproductive function as well as the relevance of the 30 kcal/kg LBM threshold in the reversal of menstrual disturbances. From this third study, we hoped to gain an understanding of the practical use of EA as a variable in reversing reproductive disturbances and whether a value of 30 kcal/kg LBM can be a useful tool in a field setting for monitoring reproductive status.

The internal and external pressures placed on exercising women to maintain low body weight or lean physique in order to improve performance have been associated with an energy deficiency and menstrual disturbances (171). Other than a recent report in high school athletes (98), no studies to date have examined the prevalence of an energy deficiency in exercising women. We do however know that almost half of exercising women experience subtle menstrual disturbances and one third might be amenorrheic depending on their sport (64). In order to quantify energy balance, both EI and the
components of total daily energy expenditure must be estimated. Characterizing endocrine abnormalities such as luteal phase defects and anovulation (68) is difficult as these reproductive disturbances must be detected with repeated hormonal measurements (62, 64). Alternatively, several indices of energy status such as EA, resting energy expenditure, resting energy expenditure ratio, metabolic hormones, and body composition have been used to corroborate an energy deficiency and the risk of menstrual disturbances (58, 84, 144). Despite the number of methods available to assess energy and menstrual status, most of these methods are not practical or feasible to exercising women. In addition, no studies to date have examined which of these methods best predicts an energy deficiency and menstrual disturbance in exercising women. In order to address these gaps in the literature, we conducted a cross sectional study to examine which indices of energy status best predicts an energy deficit and menstrual disturbance in trained exercising premenopausal women. Using a number to techniques to assess energy and menstrual status in this fourth study, we hoped to gain an understanding of not only which measure is the best but also how these measures compare to one another. The overall goal of this forth study is to provide practical and translational information regarding the best method to assess energy and menstrual status to clinicians, coaches, athletic trainers, exercise physiologists, nutritionists, and athletes to help identify exercising women at risk for the Female Athlete Triad.

When taken together, the overall goal of these four studies is to help increase our understanding of EA, defined as EI minus EEE relative to kilograms of LBM, as a tool for assessing energy and menstrual status in a field setting and consequently its usefulness in helping to identify exercising women at risk for the Female Athlete Triad.
Chapter 2
Review of Literature

What is the problem?

The Female Athlete Triad (Figure 1-1) is a syndrome of three interrelated conditions: low energy availability (EA) (without or without disordered eating), amenorrhea, and osteoporosis (171). The expansion of this syndrome to include a fourth component, cardiovascular risk, has been suggested (68, 262). These conditions, either alone or in combination, pose significant health risks to exercising females (171). The Female Athlete Triad most often develops as a result of internal and external pressures placed on females to maintain low body weight or lean physique in order to improve performance (14).

Figure 1-1. Components of the Female Athlete Triad.
Energy Availability

EA is the amount of dietary energy intake remaining after exercise training for all other metabolic processes and has been operationally defined by Loucks et al. (144) as dietary energy intakes (EI) minus exercise energy expenditure (EEE) relative to kilograms of lean body mass (LBM) (EI-EEE/kg LBM). Laboratory studies indicate that negative metabolic, reproductive, and bone related changes occur when EA drops below 30 kcal/kg LBM (103, 144). Athletes at the greatest risk for low EA are those who restrict EI, exercise for long periods of time, and limit their food choices (171). No studies to date have examined the prevalence of low EA (<30 kcal/kg LBM) or how the risk of low EA changes across a season in female athletes who have been shown to consume low EI (40) relative to high EEE (213). In the few studies that have assessed EA in free living exercising females, a wide range of values have been reported (62, 98, 134, 196, 204). In addition, EA has been defined inconsistently and measured using a variety of methods. For example, using EI, training mileage, and fat free mass from several studies of female amenorrheic runners, Loucks et al. (134) estimated EA values ranging from 12 to 29 kcal/kg FFM in these female athletes. Schaal et al. (204) observed mean EA levels below 30 kcal/kg BM in both endurance trained amenorrheic and eumenorrheic female athletes. In addition, Reed et al. (196) observed a mean EA value of 42 and 29 kcal/kg FFM in exercising females with ovulatory menstrual cycles and amenorrhea, respectively (196). To date, only one study has examined the prevalence of low EA, defined as EI minus EEE relative to kilograms of LBM, in exercising females (98). In high school athletes in a range of sports, Hoch et al. (98) found that 36% of athletes presented with an EA ≤ 45 kcal/kg LBM and 6% with an EA <30 kcal/kg LBM. Future studies are therefore needed.
to assess the prevalence of low EA in a much larger population of exercising women and whether this variable which was developed by one researcher (144) can be a useful tool in a field setting in identifying those at risk for reproductive, metabolic, and bone health outcomes that have been shown to ensue below an EA threshold of 30 kcal/kg LBM. The estimation of EI, EEE, and body composition is needed to assess EA. The inaccuracies associated with and difficulties in obtaining these measurements might not be practical or feasible for most exercising women.

**Menstrual Disturbances**

Menstrual cycle disturbances have been observed in female military recruits (120, 207-208), exercising premenopausal women (42, 45, 62, 64-65, 68, 98, 196), and high school female athletes (98, 174, 194, 229). De Souza et al. (68) proposes that a continuum of reproductive disturbances exists ranging from ovulatory, subtle presentations of luteal phase defects and anovulation to the most severe disturbances, oligomenorrhea and amenorrhea. The prevalence of subtle menstrual disturbances is difficult to determine as they are characterized by subtle endocrine abnormalities that can only be detected with repeated hormonal measurements (62, 64) or careful monitoring of basal body temperatures (242). Recently, in a large population of exercising females using daily hormone measurements, approximately half experienced subtle menstrual disturbances such as luteal phase defects and anovulation, and one third were suggested to be amenorrheic depending on their sport (64). Negative clinical outcomes such as lower bone mineral density (66), higher injury rates (182), disordered eating (84, 241), altered vascular function (176-177), reductions in resting metabolic rate (124, 168), and
suppressed metabolic hormones (58) have all been documented in females with exercise associated menstrual disturbances.

**Bone Health**

The prevalence of poor bone health in exercising females is dependent upon age, risk factors, and clinical criteria utilized to assess bone health (39, 63, 66, 68, 174, 185). Two and three dimensional imaging techniques as well as bone turnover markers can be used to assess bone health (66, 72, 103, 263-264). The World Health Organization defined osteopenia as bone mass that ranges between 1.0 and 2.5 SD below the mean normal peak bone mass. Osteoporosis is defined as bone mass that is 2.5 SD below the normal peak bone mass. The prevalence of osteoporosis and osteopenia in exercising females is approximately 6 and 48%, respectively (67-68). A recent report showed that the prevalence of low bone mineral density greater than 1.0 SD below the mean is 30% in sedentary high school female students (87). Three dimensional imaging techniques such as MRI, quantitative computed tomography (QCT) and peripheral QCT (pQCT) can be used to assess bone geometry, volumetric bone mineral density, and bone strength which two dimensional imaging techniques such as dual energy x-ray absorptiometry (DXA) cannot (72). Using QCT, hypoestrogenism in amenorrheic athletes has been shown to have a detrimental effect on spinal volumetric bone mineral density (BMD) (239). Moreover, a history of amenorrhea has been shown to compromise spinal bone mineral content and area BMD in female gymnasts (71). Low BMD increases the risk for post menopausal fractures later in life and stress fractures (169), the latter being a common problem for females athletes (151, 266). A number of studies have examined two aspects
of the Female Athlete triad such as bone health and menstrual irregularities (39, 63, 66, 70, 185), disordered eating and menstrual irregularities (57, 84, 196, 211), or bone health and disordered eating (11, 153, 241). Fewer studies have examined the prevalence of all three components (13, 42, 99, 120, 166, 174, 224). For example, in high school female athletes in whom the prevalence of menstrual irregularities, eating disorders, and low BMD were examined, 6% exhibited two components of the Female Athlete Triad and one athlete exhibited all three components (174). In competitive female distance runners, 6% of the oligomenorrheic/amenorrheic runners were osteoporotic whereas 48% were osteopenic. Disordered eating was associated with both menstrual irregularities and low BMD (42). Taken together, one or more components of the Female Athlete Triad pose significant health risks to exercising women and a large proportion of these exercising women are at risk for these negative clinical outcomes. Exercise has been shown to produce a number of positive health outcomes such as increased cardio respiratory fitness, cognitive function, and strength (123, 155). Early detection and prevention of the Female Athlete Triad will help to promote these positive outcomes associated with exercise and thus prevent the negative clinical outcomes associated with the Triad.

Treatment Options and Future Directions

Little is known about the clinical management of the Female Athlete Triad (72). Several investigators have suggested that the treatment and prevention of the Female Athlete Triad should include multiple specialists such as team clinicians, registered dieticians, mental health practitioners, coaches, athletic trainers, and exercise physiologists (158, 171). Increasing EA through diet and exercise modification is
recommended as the first line of non-pharmaceutical treatment for the Female Athlete Triad (72, 171). Other suggested treatment strategies include but are not limited to prescribing oral contraceptives, leptin administration, bisphosphonates, and antidepressants (37, 72, 262). The evidence supporting the use of these treatments remains controversial and may cause long term deleterious health effects (72). Recently, in an attempt to evaluate the risk of the Female Athlete Triad in collegiate female athletes, Hoch et al. (157) determined that the current pre participation forms used by NCAA Division I universities does not effectively screen for the Female Athlete Triad (157). Future studies are needed to develop and identify accurate yet practical tools to assess the risk of the Female Athlete Triad in a field setting. EA as a variable might be such a useful tool and thus help to prevent the associated negative health outcomes such altered vascular function (176-177), reducing in resting metabolic rate (124, 168), and suppression of metabolic hormones (58) that have been documented in exercising women with menstrual disturbances. A number of studies in both exercising females and mammalian species have demonstrated the physiological relevance of EA in the neuroendocrine regulation of reproductive function (32, 137, 178, 188-190, 193, 210, 246, 256-260).

**Neuroendocrine regulation of the menstrual cycle.**

Normal reproductive function in a variety of mammalian species is dependent upon the pulsatile release of gonadotropin-releasing hormone (GnRH) from the arcuate nucleus of the hypothalamus (191). GnRH in turn regulates the release of the pituitary gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) which
subsequently stimulates ovarian function (191). The classic studies of Knobil and colleagues (117, 191) and Spies et al. (175) with female rhesus monkeys helped to elucidate the anatomical structures involved in the neuroendocrine regulation of the menstrual cycle. Krey et al. (117) was the first to deduce that the sites of the central components of the neuroendocrine system which control the tonic and surge section of the gonadotropin hormones may be found within the medial basal hypothalamic hypophysial unit (116). In an attempt to elucidate the role of the preoptic anterior hypothalamic area found within the medial basal hypothalamus in reproductive function, Norman et al. (175) later discovered that destruction of the ventral preoptic anterior hypothalamic area blocks spontaneous ovulation and compromises the ability of the hypothalamic-hypophyseal axis to release LH in response to estrogen. Following these experiments (117, 175), Knobil and colleagues (191) confirmed that the arcuate region of the medial basal hypothalamus is the primary structure mediating the hypothalamic control of gonadotropin secretion. The intermittent release of GnRH by the hypothalamus plays a permissive, but necessary component in the neuroendocrine regulation of the menstrual cycle (111). It is the ovaries that are the principle timer of primate menstrual cycles in that estradiol controls gonadotropin secretion by acting directly on the pituitary gland.

GnRH is released in a pulsatile manner into the portal circulation about once per hour (110) which causes hourly surges in the release of the gonadotrophs, FSH and LH. The effects of LH and FSH on the ovaries vary during different phases of the menstrual cycle. The menstrual cycle which regularly spans 26 to 35 days in length (62, 64) can be divided into two phases: the follicular and luteal phase. The average duration of the
follicular and luteal phase is 15 and 14 days, respectively; however, marked variation in these phase lengths have been reported (242).

**Follicular Phase**

The follicular phase begins on the first day of menses (64). During the early through mid follicular phase, serum levels of FSH and LH rise which initiates preovulatory follicular development. The rise in FSH concentrations of approximately 30 to 50% stimulate follicular growth through the induction of granulosa cell proliferation (265). The elevated FSH concentrations stimulate the recruitment of a cohort (6-20) of pre antral follicles in the ovaries. In addition, granulosa cells acquire LH receptors in response to this FSH stimulation. FSH and estrogen exhibit a reciprocal relationship in that while FSH concentrations are elevated, those of estrogen are low (265). Several days prior to the mid cycle gonadotropin surge, serum estrogen concentrations begin to rise as the maturing follicle emerges. The increase in estrogen leads to a progressive fall in FSH concentrations due to the negative feedback action of estrogen on gonadotropin secretion (106). FSH stimulates the induction of aromatase and LH receptors on the granulose cells. Aromatase is needed to convert androgens to estrogens. The follicle produces estrogen which suppresses FSH secretion below a level that is needed to sustain the development of less mature follicles. These less mature follicles will thus undergo atresia.

Estrogen secreted from the granulosa cells of the follicle acts in an autocrine fashion to increase the number of receptors for estrogen and FSH in the follicle. As the number of estrogen receptors in the follicle increases, more estrogen is retained in the follicle, and follicular estrogen concentrations rise. The increased estrogen
concentrations stimulate further GnRH secretion from the hypothalamus as well as FSH and LH from the anterior pituitary, by way of positive feedback. Both the theca and granulosa cells are needed to produce the elevated estrogen concentrations needed to stimulate the LH surge which is essential for ovulation to occur (27). The theca cells take up cholesterol and produce androgens, but they do not contain aromatase needed to produce estrogen. The granulosa cells have aromatase, but lack the enzymes (17α-OH hydroxylase and 17,20 desmolase) needed to produce androgens. According to the “two-cell type” theory of ovarian steroid synthesis (27, 214), progesterone diffuses from the granulosa cells into the circulation to the theca cells where it is converted to androstenedione. Androstenedione diffuses back into the circulation to the granulosa cells where through the action of 17β-hydroxyprogesterone or aromatase is converted to estradiol. Within 24 to 36 hours after peak estradiol concentrations are attained (>200 pg/mL) (216), a LH surge lasting 48 to 50 hours occurs (100, 183). The LH surge stimulates the release of the oocyte from the dominant follicle.

**Luteal Phase**

Following the LH surge and release of the oocyte from the follicle, the granulosa cells undergo chemical changes to form the corpus luteum. This process is known as luteinization (27). Differentiation and growth of the corpus luteum is supported by progesterone. The corpus luteum produces both estrogen and progesterone. During the luteal phase, these hormones inhibit the release of GnRH from the hypothalamus and consequently FSH and LH from the anterior pituitary. If the ovum is not fertilized, the corpus luteum begins to degenerate within approximately 7 to 8 days following
ovulation. The demise of the corpus luteum leads to a decrease in estrogen and progesterone production which causes hemorrhagic changes in the uterine endometrium resulting in the onset of menses. The decline in these steroid hormones, estrogen and progesterone, also removes the negative feedback at the level of the hypothalamus and anterior pituitary. Consequently, LH and FSH concentrations begin to rise and stimulate the growth of another set of follicles in the ovaries which marks the beginning of the follicular phase of the next menstrual cycle (216).

The role of energy availability in the neuroendocrine regulation of the menstrual cycle

When energy is limited, animals preserve the activities essential for survival such as basic cellular functions, locomotion for foraging, and thermoregulation while other less crucial functions such as growth, body fat stores, and reproductive function are sacrificed. When energetic conditions improve, reproductive function can be resumed (246). The neural mechanisms controlling the release of gonadotropin-releasing hormone respond to short and long term changes in EA (245-247). A number of experiments using various animals species support the role of EA defined as EI minus EEE relative to kilograms of LBM in the regulation of reproductive function. For example, LH pulses were shown to resume immediately after a single meal in food restricted rats, gilts, and pre-pubertal heifers before changes in body weight or fat content occurred (246). In addition, Wade and colleagues (246) demonstrated that food deprivation on days 1 and 2 of the estrous cycle in Syrian hamsters blocks estrous behavior and ovulation in approximately 80% of the hamsters tested. The period of food deprivation inhibits
follicular development, decreases plasma estradiol, and inhibits the LH surge that occurs on the evening of day 4 in ad lib-fed hamsters (246).

The causal role of EA in the neuroendocrine regulation of reproductive function has also been demonstrated in several studies with humans. In the 1970’s, athletic coaches suspected that the occurrence of amenorrhea was greater in females athletes who trained heavily. To address this question, Feicht and colleagues (77, 202) administered questionnaires to collegiate female runners to examine the relationship between the incidence of amenorrhea and training characteristics. A positive relationship between the incidence of amenorrhea, defined as three or less menstrual periods in one year, and weekly training volume was observed (77, 202). Following these initial investigations (77, 202), a number of prospective observational studies examined the association between exercise training and reproductive function (30, 193). These studies (30, 193) reported disturbed follicular development, luteal phase defects, and lower estrogen and progesterone concentrations in recreational female athletes (30). In addition, abnormal menstrual cycles, 32 of 48, in 14 premenopausal women who maintained normal menstrual cycle length during marathon training were observed (193). Taken together, the findings from the above mentioned studies support the role of optimal EA in the neuroendocrine regulation of the menstrual cycle.

**Short Term Studies**

Several short term studies manipulating EI and EEE (144, 256-257, 260) or EI alone (137, 178, 188-190, 210) support the role of EA in the neuroendocrine regulation of reproductive function. For example, decreases in FSH (26), estrogen (31), and
progesterone (31) concentrations as well as irregular menstrual cycles (26) were observed in females who participated in several weeks of aerobic exercise training. Through decreases in EI alone, a range of menstrual disturbances including decreased estrogen concentrations, luteal phase defects, anovulation, amenorrhea, and decreased LH pulses have been observed (35, 187-190). Similar to these findings, low EA conditions produced disruptions in LH pulses in trained women (260) and a number of cross sectional studies documented reduced LH pulses in eumenorrheic (50), oligomenorrheic (240), and amenorrheic (50, 142, 240) runners and exercising women with anovulatory menstrual cycles (187). Specifically, the most severe reductions in LH pulsatility have been observed in amenorrheic (142, 240) runners. Less severe reductions in LH pulsatility have been observed in exercising women with eumenorrhea (50), anovulatory menstrual cycles (187), and oligomenorrhea (240).

**Prospective Studies**

In the only prospective exercise training and weight loss intervention in humans to date to examine changes in menstrual cycle characteristics in response to an abrupt exercise training intervention in previously sedentary women, abnormal luteal function, delayed menses, and loss of LH surge were observed (32). Delayed menses and loss of LH surge were more prevalent during the second of a total of two menstrual cycles suggesting that menstrual disturbances progress in severity when an energy deficit is maintained (32). Exposure to abrupt exercise training has been shown to impact luteal function, regardless of whether the exercise was limited to the follicular or luteal phase (32, 256).
Using a monkey model, Williams et al. (257-258) provided evidence for the causal role of EA in reproductive function. Williams et al. (257) first examined the changes in reproductive hormones and menstrual cyclicity in eight cynomolgus monkeys in response to a gradual strenuous exercise training regimen. EI was held constant while the monkeys progressed from being sedentary to running approximately 12 kilometers per day. Decrease in FSH concentrations in the late luteal phase were followed by significant decreases in LH and progesterone, and an increase in follicular phase length preceded the development of amenorrhea which occurred within 14 months (257). Williams et al. (258) then used a prospective design to examine the impact of increased EI in four of these cynomologus monkeys who had recently completed the strenuous exercise training intervention resulting in amenorrhea. Increased EI while maintaining strenuous exercise resulted in the resumption of menses and significant increases in LH, FSH, estradiol, progesterone, and T\textsubscript{3} concentrations in all four monkeys. The rate of recovery of menstrual cyclicity was found to be inversely related to EI ($r = -0.98, p < 0.024$) (258). In the two animals that recovered more quickly, LH surge within 12 and 16 days, EI during the period of refeeding averaged 163% and 181% of their EI while they were amenorrheic. In the animals that recovered more slowly, 50 and 57 days, average EI was 138% and 141% above their EI while they were amenorrheic (258). These well controlled and eloquent studies clearly demonstrate the causal role of EA on reproductive function (257-258). These studies in addition to that of Loucks et al. (144) also show that exercise training, beyond the impact of its energy cost, has no suppressive effect on reproductive function in females (145, 258).
Impact of Improved EA

Several studies in animals (246, 258) and humans (8, 73, 83, 86, 115, 163) have documented the effectiveness of either improved EI (83, 86, 163, 258), decreased EEE (249), or the manipulation of both (73, 115) to improve indices of reproductive function. For example, resumption of menses occurred during times of rest from injury in female ballet dancers who were previously amenorrheic (249). Resumption of menses also occurred in several amenorrheic female athletes following a 15 to 20 week diet and exercise intervention designed to improve energy balance. Athletes decreased EEE by adding 1 rest day to their training schedule and increased EI by adding one sport nutrition supplement to their total daily EI (73, 115). Using a non-human primate model, increases in gonadotropin hormones, ovarian steroids, and resumption of menses were documented in cynomologus monkeys who increased EI while maintaining strenuous exercise training (258). Changes in body weight are generally associated with alterations in reproductive function resulting from changes in EI and/or energy expenditure as observed in amenorrheic female athletes (73, 115, 249) and monkeys (258) who resumed menses; however, it is important to note that body weight is not always an accurate reflection of changes in energy balance (257, 259) or EA as subtle changes in energy status i.e., - 400 kilocalorie may not result in concomitant changes in body weight. It is also possible that changes in body weight occur in response to increases in plasma volume (46) and body water stores which accompany glycogen storage (179) when energy status is unaltered.
Other Metabolic Changes Associated with Reproductive Suppression

In addition to the above reproductive changes, metabolic changes have also been shown to occur in response to low EA conditions. Several cross sectional studies have documented various metabolic disruptions including lower resting metabolic rate (58, 124, 168, 170), lower T₃ (58, 141), higher ghrelin (58-59), and higher PYY (206) concentrations in exercising females with menstrual disturbances. Moreover, short term studies inducing low EA conditions in previously sedentary females have been shown to produce decreases in T₃ (136, 138), leptin (97), glucose (144) insulin (144), and IGF-1 (144) concentrations as well as increases in cortisol (144) and growth hormone (144). In prospective studies in monkeys (257-258), decreases in T₃ concentrations occurred with the induction of menstrual disturbances. Significant increases in T₃ concentrations were later observed prior to changes in FSH, LH, and estradiol concentrations in monkeys who resumed menses in response to increased EI while maintaining strenuous exercise training (258).

Findings are conflicting regarding the role of some metabolic hormones in reproductive function. For example, elevated serum cortisol concentrations have been shown to predict longer time to resumption of menses (8). In support, decreases in cortisol concentrations were observed in amenorrheic female athletes who resumed menses following several weeks of a diet and exercise intervention (73, 115). However, Misra et al. (164) reported that anorexia nervosa patients with higher baseline cortisol concentrations might gain more fat mass with weight gain, predicting a greater chance for resumption of menses. The administration of leptin, a marker of energy balance, was shown to be an effective pharmacological treatment for amenorrhea (37). However,
circulating leptin concentrations have been shown to not distinguish menstrual status in exercising women (48) or predict resumption of menses in anorexia nervosa patients (164).

Although the above studies (8, 37, 73, 83, 86, 115, 163, 249, 258) suggest that improved energetic conditions play a role in the recovery of reproductive function, the degree of change in EA needed to induce or reverse reproductive disturbances remains unknown. These studies are also limited to due sample size (73, 115) and the use of laboratory (37, 258) or clinical (8, 83, 86) methods not practical for free living exercising women. Future studies should address these questions as well as explore the most practical and accurate tools in the field to monitor menstrual status.

**The relevance of EA**

*Physiological Relevance of EA*

According to Loucks et al. (144), EA represents the amount of EI remaining after exercise training for all other metabolic processes. Loucks et al. (144) demonstrated that during short term manipulation of EI and EEE in a controlled laboratory setting in previously sedentary females, low EA can produce negative metabolic, bone, and reproductive outcomes. In particular, low EA (<30 kcal/kg LBM) has been shown to suppress several metabolic hormones including T₃, leptin, glucose, insulin, and insulin-like growth factor-1 (144), several bone markers including estradiol, osteocalcin, and type 1 procollagen carboxy-terminal propeptide (103), and LH pulsatility (144).

These elegant studies conducted by Loucks and colleagues (97, 103, 136, 138, 144) define a critical threshold for the initiation of the suppression of gonadotropin
releasing hormone pulse generator activity; however, a number of integral questions regarding the role of EA defined as EI minus EEE relative to kilograms of LBM have yet to be examined. First, few studies (62, 98, 196, 204) have examined whether an EA of 30 kcal/kg LBM discriminates actual menstrual status. In particular, few studies (62, 98, 196, 204) have examined whether an EA of 30 kcal/kg LBM discriminates ovulatory menstrual cycles from exercise associated menstrual disturbances including luteal phase defects, anovulatory menstrual cycles, oligomenorrhea, and amenorrhea. Second, no studies to date have examined whether more severe menstrual disturbances are associated with more severe declines in EA. Last, no studies to date have examined the role of EA in the reversal of menstrual disturbances in exercise and or diet related amenorrhea or oligomenorrhea. All of these questions have not been addressed when EA has been determined using methods available outside the laboratory setting in free living participants.

Practical and Translational Relevance

A few studies have documented the EA of exercising women (62, 98, 134, 196, 204) and several more (118, 121, 170, 232-233, 261) have reported EI, EEE, and body composition which allows for the estimation of EA to be made. In these studies, EA has been defined inconsistently and measured using a variety of methods. For example, using EI, training mileage to estimate EEE, and fat free mass, EA values ranging from 12 to 29 kcal/kg FFM have been estimated from several studies of amenorrheic runners (134). In another report, mean EA values less than 30 kcal/kg LBM in both trained amenorrheic and eumenorrheic female athletes who self-reported menstrual status were observed
This finding differs from the greater EA observed in exercising women with ovulatory menstrual cycles when compared to those with amenorrhea when menstrual status was confirmed with daily urinary metabolites E1G and PdG (196). Below is a comprehensive table which contains all of the studies that have measured EA or its components in exercising women (See section “What studies have measured EA or its components?”). All of these studies except one (233) (Table 1-1, Figure 1-2), have documented low EA values (<30 kcal/kg LBM, <30 kcal/kg FFM) in exercising women with menstrual disturbances. Studies are conflicting regarding the EA of eumenorrheic exercising women as some (118, 170, 196, 232-233) have documented higher EA values (>30 kcal/kg LBM, >30 kcal/kg FFM) while others (62, 121, 204, 261) have documented low EA values (<30 kcal/kg LBM, <30 kcal/kg FFM). These findings suggest that EA may be a practical tool for exercising women to assess their energy status and consequently risk for the Female Athlete Triad such that lower EA values would be suggestive of a greater risk for the Triad. The above findings should however be interpreted with caution as these studies are limited due to low sample size (204) and inconsistency of methods used to assess EA, body composition, and menstrual status (62, 98, 134, 196, 204).
Table 2-1. Energy availability of free living exercising females with varying menstrual status

<table>
<thead>
<tr>
<th>Study</th>
<th>Subject Characteristics</th>
<th>Age</th>
<th>Sample Size</th>
<th>Energy Availability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reed et al. 2011 (196)</td>
<td>Exercising ovulatory women</td>
<td>18-35</td>
<td>13</td>
<td>42.1±9.2 kcal/kg FFM</td>
</tr>
<tr>
<td></td>
<td>Exercising amenorrheic women</td>
<td></td>
<td>12</td>
<td>28.8±11.5 kcal/kg FFM</td>
</tr>
<tr>
<td>Lagowska et al. 2010 (118)</td>
<td>Athletes with regular menstrual cycles</td>
<td>18-21</td>
<td>8</td>
<td>36.0 kcal/kg FFM*</td>
</tr>
<tr>
<td></td>
<td>Athletes with menstrual disorders</td>
<td></td>
<td>12</td>
<td>24.7 kcal/kg FFM*</td>
</tr>
<tr>
<td>Hoch et al. 2009 (98)</td>
<td>High school female varsity athletes</td>
<td>13-18</td>
<td>80</td>
<td>n=51 (64%), &gt;45 kcal/kg LBM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n=24 (30%), &gt;30 and ≤54 kcal/kg LBM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n=5 (6%), &lt;30 kcal/kg LBM</td>
</tr>
<tr>
<td>Schaal et al. 2009 (204)</td>
<td>Endurance trained eumenorrheic women</td>
<td>26-36</td>
<td>6</td>
<td>29 ± 4.8 kcal/kg LBM</td>
</tr>
<tr>
<td></td>
<td>Endurance trained amenorrheic women</td>
<td></td>
<td>6</td>
<td>18 ± 6.6 kcal/kg LBM</td>
</tr>
<tr>
<td>Loucks, AB. 2007 (134)</td>
<td>Amenorrheic runners</td>
<td></td>
<td></td>
<td>12-29 kcal/kg FFM*</td>
</tr>
<tr>
<td>Tomten and Hostmark 2006</td>
<td>Runners with regular menstrual function</td>
<td>17-40</td>
<td>10</td>
<td>58.8 kcal/kg LBM*</td>
</tr>
<tr>
<td>(233)</td>
<td>Runners with irregular menstrual function</td>
<td></td>
<td>10</td>
<td>40.0 kcal/kg LBM*</td>
</tr>
<tr>
<td>Thong et al. 2000 (232)</td>
<td>Eumenorrheic recreational athletes</td>
<td>20-25</td>
<td>13</td>
<td>30.1 kcal/kg LBM*</td>
</tr>
<tr>
<td></td>
<td>Eumenorrheic elite athletes</td>
<td></td>
<td>8</td>
<td>29.5 kcal/kg LBM*</td>
</tr>
<tr>
<td></td>
<td>Amenorrheic elite athletes</td>
<td></td>
<td>5</td>
<td>15.7 kcal/kg LBM*</td>
</tr>
<tr>
<td>Kopp-Woodroffe et al. 1999</td>
<td>Amenorrheic athletes</td>
<td>18-35</td>
<td>4</td>
<td>25.1±4.8 kcal/kg FFM*</td>
</tr>
<tr>
<td>(115)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>De Souza et al. 1998 (62)</td>
<td>Exercising ovulatory women</td>
<td>18-36</td>
<td>24</td>
<td>28.7 kcal/kg LBM* 31.7 kcal/kg LBM*</td>
</tr>
<tr>
<td></td>
<td>Exercising women with LPD</td>
<td></td>
<td>21</td>
<td>22.3 kcal/kg LBM*</td>
</tr>
<tr>
<td></td>
<td>Exercising women with Anov</td>
<td></td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Laughlin and Yen 1996</td>
<td>Eumenorrheic athletes</td>
<td>18-36</td>
<td>8</td>
<td>18.2 kcal/kg LBM*</td>
</tr>
<tr>
<td>(121)</td>
<td>Amenorrheic athletes</td>
<td></td>
<td>8</td>
<td>23.9 kcal/kg LBM*</td>
</tr>
<tr>
<td>Wilmore et al. 1992 (261)</td>
<td>Eumenorrheic distance runners</td>
<td>18-29</td>
<td>5</td>
<td>17.4 kcal/kg FFM*</td>
</tr>
<tr>
<td></td>
<td>Amenorrheic distance runners</td>
<td></td>
<td>8</td>
<td>18.0 kcal/kg FFM*</td>
</tr>
<tr>
<td>Myerson et al. 1991 (170)</td>
<td>Amenorrheic runners</td>
<td>18-34</td>
<td>6</td>
<td>27.5 kcal/kg FFM*</td>
</tr>
<tr>
<td></td>
<td>Eumenorrheic runners</td>
<td></td>
<td>9</td>
<td>32.6 kcal/kg FFM*</td>
</tr>
</tbody>
</table>

*, EA values are estimated from the reported mean dietary energy intake, exercise energy expenditure, and body composition; †, estimated values by Loucks et al. (134)
LBM, lean body mass; FFM, fat free mass; BW, body weight; LPD, luteal phase defects; Anov, anovulatory menstrual cycles.

Figure 2-1. Energy availability of free-living exercising females of varying menstrual status. 1, Reed et al. (196); 2, Lagowska et al. (118); 3, Schaal et al. (204); 4, Tomten and Hostmark (233); 5, Thong et al. (232); 6, Kopp-Woodroffe et al. (115); 7, De Souza et al. (62); 8, Laughlin and Yen (121); 9, Wilmore et al. (261); 10, Myerson et al. (170).
The physiological importance of EA in both the induction and reversal of reproductive dysfunction has been demonstrated (257-258). However, an important practical consideration that has not been adequately explored is the magnitude of change in EA necessary to either induce or reverse menstrual disturbances in exercising women. Understanding whether a particular threshold of EA exists below which reproductive disturbances ensue, or above which reproductive recovery is stimulated, is also a key translational question that must be addressed for correctional nutritional strategies to be developed. This is especially important for special populations of women susceptible to energetically related reproductive dysfunction such as female military recruits (120, 207-208), exercising women (57-58, 64, 68, 98, 196), and or women with disordered eating behaviors (42, 57, 84, 241).

*Advantages and Disadvantages of EA Measurement*

EA, calculated as EI minus EEE relative to kilograms of LBM (144), can be measured with relative ease depending on the methods or tools available to exercising women. For instance, the compendium of physical activities (4, 6) to estimate EEE, online nutritional analysis programs to calculate EI, and bioelectrical impedance to measure LBM will permit the measurements of EA with minimal cost (134). Methods with greater precision such as heart rate monitors ($80-250, Polar Electro) and DXA scans (research or clinical machine) are available to assess the components of EA, but the cost and ease of accessing these tools might prevent their use. If eventually EA is used in the field, the use of a cut off value will be associated with misclassification at times because the error associated in measuring the components of EA i.e., EI, EEE, and LBM.
In particular, concerns with under reporting (23, 261), misreporting (23-24, 150, 261), and eating behaviors (234) associated with assessing EI as well as the errors associated with various methods to assess EEE and LBM can all impact the precision of the EA measurement. Below is a more detailed description of the limitations of these methods (See section “What is the best tool to identify an energy deficiency and/or menstrual disturbance?”). Future studies are therefore needed to assess the variability in the EA measurement.

The components of 24 hour energy expenditure (TDEE) include resting energy expenditure, thermic effect of food, and physical activity energy expenditure. Resting energy expenditure accounts for approximately 60 to 70% of TDEE, thermic effect of food accounts for approximately 10% of TDEE, and physical activity energy expenditure accounts for approximately 20 to 35% of TDEE (130). Physical activity energy expenditure encompasses both purposeful energy expenditure and non-exercise activity thermogenesis (128). The calculation of EA, defined as EI minus EEE relative to kilograms of LBM, does not account for resting energy expenditure, the thermic effect of food, or non-exercise activity thermogenesis which can represent approximately 80% of total daily energy expenditure (130). Examples of non-exercise activity thermogenesis include laundry, walking, and reading a book.

Reductions in resting energy expenditure have been observed in female athletes with menstrual disturbances (58, 124, 168, 170). Non-exercise associated thermogenesis can vary as much as 2000 kcal/d and both increases and decreases in non-exercise activity thermogenesis have been detected in a state of positive and negative energy balance, respectively (125, 129, 199). Changes in these parameters of energy balance
when combined can account for approximately 80% of total daily energy expenditure are not accounted for in the calculation of EA. Thus whether EA accurately reflects a positive or negative energy balance and is a useful field tool to assess energy status when certain components of total daily energy expenditure i.e., non-exercise activity thermogenesis are not controlled under rigorous laboratory conditions remains to be determined.

**What studies have measured EA or its components?**

Several studies have documented the EA, or provided the EI, EEE, and body composition to estimate EA, of free living exercising females with varying menstrual status (Table 1-1) (62, 98, 115, 119, 121, 134, 170, 196, 204, 232-233, 261). The methods however used to assess EI, EEE, and body composition vary considerably between the studies presented in Table 1-1. In addition, the criteria used to categorize menstrual status also varies between these studies (62, 98, 115, 119, 121, 134, 170, 196, 204, 232-233, 261). These inconsistencies may account for the large range of EA values observed in these exercising females with varying menstrual status. For example, EA ranging from 17.4 to 58.8 kcal/kg LBM (233, 261) and 12 to 29 kcal/kg FFM (134) has been observed in eumenorrheic and amenorrheic exercising females, respectively.

Of all the studies presented in Table 1-1, De Souza et al. (62) and Reed et al. (196) are the only two that have used daily measures of urinary estrone-1-glucuronide (E1G), pregnanediol-3-glucuronide (PdG), and luteinizing hormone (LH) profiles along with self-reported menstrual histories to accurately assess menstrual status (68). Several investigators used clinical documentation (62, 98, 196, 204) and/or reproductive
hormones (62, 98, 115, 121, 196, 233, 261) in addition to self reported menstrual histories to confirm menstrual status. For example, hormonal analyses to rule out organic causes of functional hypothalamic amenorrhea including hyperprolactinemia (62, 98, 121, 196), thyroid (62, 98, 115, 121, 196) and adrenal (121) diseases, and hyperandrogenism (121, 196) were performed.

The remaining studies (170, 204, 232) have primarily used self-reported menstrual histories to categorize reproductive status. For example, eumenorrhea has been defined by some investigators as menstrual cycles of 25 to 42 days for 3 years (170), menstrual cycles of 26 to 35 days for at least 3 months (232), 10 to 12 or more menstrual cycles in the last year (118, 204), or simply no menstrual dysfunction after establishing menarche (261). Menstrual cycle length is not an accurate indicator of ovarian function (64, 68). Thus, the EA of these eumenorrheic women may in fact be representative EA values of women with luteal phase defects, anovulatory menstrual cycles, or oligomenorrhea (62, 64). Amenorrhea has been defined as an absence of menses for at least 6 months (118, 121, 170, 232) and less conservatively as no menses within the last 3 months or no more than 3 to 4 periods in the last year (115, 204, 261).

Self-reported diet logs spanning 3 to 7 days are commonly used practices to assess EI (234). Most studies (98, 196, 233, 261) presented in Table 1-1 used self-reported 3 day diet logs (2 weekdays, 1 weekend day) to assess EI. Participants were often trained by a registered dietician how to accurately record food intake and encouraged to weigh all foods and beverages (98, 196, 233). Weighed diet records are considered the gold standard among dietary assessment methods (20). Others studies (62, 118, 121, 170, 204, 232) used self-reported 7 day diet logs to assess EI. Kopp-Woodroffe
et al. (115) used 7 day weighed diet logs and De Souza et al. (62) provided participants with techniques to estimate food portion sizes with the use of food models. Food models have been shown to improve the accuracy of self-reported diet logs (25). However, collection of 3 to 4 day instead of 7 day diet logs reduces participant burden and improves compliance (93). Please see below for a comprehensive discussion regarding the limitation and issues with under reporting in exercising women (See section “What is the best tool to identify an energy deficiency?”).

A number of methods including exercise diaries, heart rate monitors, indirect calorimeters, and accelerometers have been used to estimate EEE. Reed et al. (196), Hoch et al. (98), and Wilmore et al. (261) used the Ainsworth compendium (5-6) to convert activities listed on exercise logs to energy equivalents. Lagowska et al. (118) used a predicted resting energy expenditure equation and physical activity level (PAL) appropriate for the exercise listed on physical activity questionnaires to estimate EEE (76). Estimating EEE from exercise logs has been shown to overestimate energy expenditure by approximately 8% when compared to doubly labeled water (47). Several studies (170, 204, 233) used the relationship between heart rate and oxygen uptake obtained during exercise sessions in the laboratory using indirect calorimetry to calculate training related EEE in a field setting. Indirect calorimetry has been shown to measure EEE within ±10% when compared to doubly labeled water (2). Please see below for a more comprehensive discussion on the use of heart rate monitors in assessing EEE (See section “What is the best tool to identify an energy deficiency”). De Souza et al. (62) used energy expenditure from a Caltrac accelerometer to compute EEE. This accelerometer has been shown to estimate EEE within -10 to +14% when compared to indirect
calorimetry (226). Lastly, Laughlin and Yen (121), Kopp-Woodroffe et al. (115), and Thong et al. (232) used prospective 7 day activity records to assess EEE; however, the methods used to convert activities listed on exercise logs to kilocalories were not provided.

In addition to the various measures that have been used to assess EI and EEE in the studies presented in Table 1-1, dual energy x-ray absorptiometry (DXA) (115, 121, 196, 204), underwater weighing (170), bioelectrical impedance (118) and skin fold measurements (62) methodologies have all been used to assess body composition. In comparison to the gold standard four compartment model, DXA and underwater weighing are acceptable methods for assessing body composition. The DXA and underwater weighing have been shown to estimate percent body fat within 3.5% and -3.3 to 3.1%, respectively, in comparison the four compartment model; however, there is considerable inter-individual variability in using skin fold measurements (41).

The differences in the above methodologies and their associated reliability and validity for assessing EI, EEE, and body composition should be considered when reviewing the findings presented in Table 1-1.

What do we currently know about the prevention and treatment of low EA?

Findings from recent studies examining the coupling between EI and EEE (101, 108, 171, 230, 253-254) suggest that inadvertent under eating leads to low EI. For example, Hubert et al. (101) examined the impact of inducing an energy deficit by altering exercise or caloric content of meals on appetite in young exercising women. Participants consumed more calories during an ad libitum lunch following a low versus
high calorie breakfast, but not more calories during an ad libitum lunch when a low calorie breakfast was followed by a 40 minute bout of moderate exercise. Whybrow et al. (254) recently examined feeding responses to increased levels of physical activity in young, lean exercising females and males. Only a 30\% compensation of the exercise induced energy expenditure, with the degree of compensation varying considerably among participants was observed. The lack of an increase in EI in response to an acute exercise induced energy expenditure i.e., inadequate compensation has been observed in young, obese, and non-obese men and women (108, 230, 253-254). The above studies (108, 171, 230, 253-254) suggest that inadvertent dietary compensation to match exercise energy may contribute to low EA in exercising women.

Disordered eating attitudes may also predispose female athletes to lower EI (171). The prevalence of eating disorders has been shown to be greater in female athletes competing in aesthetic sports (42\%) when compared to endurance (24\%), technical (17\%), and ball game (16\%) sports (225). However, an unexpected high prevalence of eating disorders (28\%) was observed among female athletes participating in ball game sports (224). Greater eating, weight, and shape concerns have been observed in elite female runners with eating disorders (102). Higher drive for thinness, dietary cognitive restraint, and body dissatisfaction are common eating attitudes of individuals at risk for disordered eating and have been observed in energy deficient exercising premenopausal women (42, 57, 84, 102, 196, 241). These higher scores (42, 102, 196) may indicate that exercising women, owing to their desire to remain at a lower weight, choose to consume fewer kilocalories which consequently may contribute to low EA.
Eating behaviors such as EI of particular meals and number of eating occasions have also been shown to impact total daily EI. For example, in one case, EI at dinner was almost twice as great in energy replete runners with normal menstrual function when compared to energy deficient runners with irregular menstrual function (233). A number of studies have noted the high frequency of eating occasions in athletes who report large EIs (34, 92). Eating a number of small meals and snacks daily might be a practical strategy that allows for increased EI as it may reduce the gastrointestinal discomfort of infrequent large meals (92). Alternatively, a reduced number of eating occasions and or EI at particular meals may contribute to low EA in exercising women.

There are a number of physiological mechanisms that promote energy homeostasis. For example, ghrelin is a peptide hormone which is produced in a distinct endocrine cell type of the stomach and gastrointestinal tract (54). It has been proposed to play a central role in short-term energy homeostasis and is a circulating peripheral orexigenic hormone known to stimulate appetite. Elevated ghrelin concentrations have been observed in energy deficit exercising women (59). PYY is a gastrointestinal peptide secreted from the endocrine L cells of the intestine in response to caloric intake. PYY plays a greater role in long term energy homeostasis and has been shown to be elevated in individuals with low BMI (205) and in energy deficient women with menstrual irregularities (206). Despite these regulatory mechanisms to promote an energy balance, EI is not always well matched to energy expenditure (235). In particular, the uncoupling of EI and energy expenditure, negative eating attitudes, and nutritional strategies that promote consuming low energy density foods may lead to lower EI compared to higher energy expenditure. These behaviors should be monitored among exercising females to
prevent low EA and the associated negative health and metabolic health consequences that have been observed during low EA conditions (68, 171). Exercising women must learn to eat by discipline instead of appetite (143). To increase EI to meet the needs of training, Burke and colleagues (33) suggest consuming small, frequent meals and snacks throughout the day, avoid excessive intake of low energy-dense and fiber-rich foods, make use of energy and nutrient dense fluids such as fortified milk drinks and liquid meal supplements.

**What is the best tool to identify an energy deficiency?**

Energy status can be measured using a direct (89) or number of indirect techniques (2). A room calorimeter is the only direct method of measuring energy expenditure. Participants are placed in a thermally-isolated chamber where dissipated heat in the form of evaporation, radiation, conduction, and convection is measured and then used to compute energy expenditure (2). A room calorimeter provides high accuracy (5.5%) under both low and high activity levels (56) and is considered the gold standard for measuring energy expenditure in the laboratory (2). This method is however time consuming, expensive, and requires a room calorimeter which makes this method of measuring energy expenditure impractical for most laboratory and all field settings (89).

*Doubly Labeled Water*

A number of indirect techniques including indirect calorimetry (2, 209), heart rate monitors (36), accelerometers (12, 226), and exercise logs (4-6) can be used to estimate energy expenditure. Doubly labeled water (DLW) is a form of indirect calorimetry. It is
considered the gold standard for measuring energy expenditure for short periods of time in free living conditions (2). Participants receive an oral dose of a weighted amount of water labelled with a hydrogen isotope (\(^2\)H, deuterium) and oxygen isotope (\(^{18}\)O). The \(^2\)H is excreted from the body as water and the \(^{18}\)O is excreted as water and carbon dioxide for up to three weeks after the initial oral dose is consumed. The isotopes are excreted in bodily fluids i.e., urine, blood or saliva and then measured using radio mass spectrometry. The difference in the elimination rates of these isotopes is proportional to carbon dioxide production. There are a number of equations that can be used to calculate carbon dioxide production i.e., \[ [r\text{CO}_2 \text{ (moles/d) } = \text{TBW} / 2 \left[ (1.041 \times k^{2}\text{H}) - (1.007 \times k^{18}\text{O}) \right] ] \] (215). The rate of carbon dioxide production is then used to estimate energy expenditure \[ [\text{energy expenditure (kcal/d)} = 3.94 / \text{RQ} + 1.10 \times 22.41 \times r\text{CO}_2] \] (209, 215). The precision of the DLW method varies between 2 and 8% (209). The high cost of isotopes and specialized equipment needed for the analysis of isotope concentrations in body fluids by mass spectrometry makes this method impractical for field measures (2).

**Indirect Calorimetry**

A current and popular method for estimating energy expenditure is indirect calorimetry. A calorimeter collects and quantifies the volume and concentrations of expired CO\(_2\) and inspired O\(_2\) (75, 186). Using the volume of these gases, energy expenditure is calculated using the Weir equation \[ [(\text{EE (kilocalories/d)} = [3.94(\text{VO}_2) + 1.11(\text{VCO}_2)] \times 1.44)] \] (252). This automated process replaces the Douglas bag method where outside air is inhaled through a mouthpiece and exhaled into a Douglas bag or Tissot tank. The volume of air in the bag or tank is measured to calculate minute
ventilation. A separate gas analyzer measures the CO₂ and O₂ concentration that is obtained from a sample of air (2). An indirect calorimeter is useful for estimating energy expenditure during long term measurements in which participants are resting i.e. resting energy expenditure or performing light exercise under controlled laboratory conditions.

Indirect calorimetry has been shown to estimate energy expenditure in adult men within 0.63±0.63% and 15.34±2.52% when compared to direct calorimetry and DLW, respectively (212). A number of portable indirect calorimeters i.e., Oxycon Mobile (Care Fusion, San Diego, CA), Aerosport KB1-C (Aerosport Inc., USA), and Cortex MetaMax3B (Cranlea, Birmingham, UK) offer the advantage of measuring energy expenditure in a field setting. Few manufactures provide data regarding their validity or reliability. In the reports that have been reported, the precision of these machines vary when compared to one another, DLW, and direct calorimeters (148, 154, 201, 243).

Using indirect calorimetry to measure resting energy expenditure, which represents 60 to 70% of total daily energy expenditure, in a controlled laboratory setting is a reliable and objective way to assess energy status (44). A number of prediction equations using factors such as age, gender, height, weight, fat mass, and fat free mass have been used to estimate resting energy expenditure (51-52, 231). De Souza and colleagues (57) operationally defined a measure to assess adaptations of prolonged energy deficiency as the ratio of measured resting energy expenditure (REE) to predicted resting energy expenditure (pREE) by the Harris-Benedict equation

\[ \text{REE} = 655.1 + 9.56(Wt) + 1.85(Ht) - 4.68(Age) \] (90), as <90%. Most studies in women with anorexia nervosa used the Harris-Benedict equation to predict REE (114, 156, 192). During periods of low body weight and prior to refeeding, a reduced ratio of measured
REE to Harris-Benedict predicted REE of 0.60 to 0.80 is often reported (114, 156, 192). De Souza et al. (57, 84) has shown that a REE:pREE less than 0.90 in exercising women with menstrual cycle disturbances (66, 84, 206). This reduced REE ratio is similar to the above mentioned findings in anorexic women. To date, the use of this ratio as a method to assess energy status has not been validated in the literature. In addition, the prediction equation to which the actual resting energy expenditure is compared could also influence the cut-off used to identify energy deficient females. For example, the Cunningham equation [REE=500+22(FFM)] (51) has been shown to provide a more accurate estimate of resting energy expenditure when determining the energy needs of male and female endurance athletes (231). An equation to accurately estimate resting energy expenditure in exercising premenopausal women with varying menstrual status has yet to be determined. Further research is therefore needed to explore whether a resting energy expenditure ratio is able to accurately assess energy and menstrual status.

Heart Rate Monitoring

During exercise there is a close linear relationship between heart rate and VO$_2$ which allows the estimation of energy expenditure to be made from heart rate (2). Several studies with exercising females have used this method to estimate energy expenditure (15, 36, 204). Studies examining the validity and reliability of heart rate monitoring in estimating energy expenditure have shown heart rate monitoring to underestimate total energy expenditure by a mean value of 1.2 (SD 6.2) % (range -11.4 to 10.6%) (p>0.05) when compared to indirect whole-body calorimetry (36). When compared to DLW, heart rate monitoring has been shown to estimate energy expenditure within -4.0 to
11.4% in females and ± 10% when males were included in the analyses (132). The optimal method to estimate energy expenditure from heart rate is known the FLEX HR method (217). With this technique, heart rate and VO₂ are measured simultaneously while participants lie down, sit, stand, and perform exercise at various intensities. This information is used to develop an individual HR-VO₂ curve which enables estimating energy expenditure from measured heart rate. This method of estimating energy expenditure from heart rate in the laboratory is both expensive and time consuming. A number of companies manufacture heart rate monitors that EEE. For example, the OwnCal feature of Polar heart rate monitors (Polar Electro Oy, Kempele, Finland) (49) has been validated for the use in calculating EEE from heart rate (36). This feature uses body weight, height, age, gender, VO₂max, individual maximum heart rate, individual heart rate in a sitting position, and heart rate during exercise to derive kilocalories from energy expenditure.

Physical Activity Questionnaires

Physical activity questionnaires can also be used to assess energy status (4-6, 28, 47, 250). The length of the recording period and detail required to estimate energy expenditure vary considerably between questionnaires (2). For example, the activity questionnaire designed by Bouchard et al. (28), requires participants to record their physical activity in 15 minute intervals over 3 days, including a weekend day. Each of the activities is then converted to a metabolic equivalent value ranging from 1 to 15. Conversely, in order to retrospectively estimate energy expenditure, participants can be asked in a structured interview format to recall time spent in sleep, light, moderate, and
hard physical activities during a previous defined period of time (250). Common 7 day physical activity records and 7 day physical activity recall questionnaires which use the Ainsworth et al. (5) compendium to estimate the energy cost of movements have been shown to overestimate total energy expenditure by 7.9±3.2 and 30.6±9.9%, respectively, when compared to DLW (47). Seven day physical activity recall questionnaires may better estimate EEE in females than males as they have been shown to estimate EEE within ±10% of the value obtained from DLW in 30% of women compared to a smaller 6% in men (250).

**Accelerometers**

Accelerometers measure segment or limb acceleration along one or multiple axes. Some common accelerometers used to estimate energy expenditure include the ActiTrainer (ActriGraph, Pensacola, FL), Caltrac (Hemokinetics, Inc.), RT3 (Stay Healthy, Monrovia, CA), and Actiheart (Respironics Co., Bend, OR). Most accelerometers use equations that have been developed for certain activities to convert counts detected through movements to energy expenditure. Accelerometers are small, non-invasive, and provide an objective record of movement for short or long periods of time in a field setting. However, a number of limitations are associated with this method of estimating energy expenditure. For example, motion artifacts from activities such as driving a car or riding a train (7), improper placement, inability to capture upper body movements with hip worn accelerometers or lower body movements with arm worn accelerometers may all contribute to over or under estimating energy expenditure (248). In fact, several studies (19, 113, 173, 226) indicate that accelerometers may be of limited
use in estimating energy expenditure. For example, the Caltrac has been shown to
overestimate energy expenditure in runners (+14%) and walkers (+19%) but
underestimate energy expenditure in steppers (-10%) (226). Several accelerometers
including the ActiGraph 7164, ActiReg, ikcal, and SenseWear Pro have been shown to
underestimate total energy expenditure by 5 to 21% when compared to indirect
calorimetry (19). In addition, the RT3 has been shown to underestimate activity related
energy expenditure by 485 kcal/d (15%) when compared to doubly labeled water (149).

Recent advances in accelerometer research have led to the development of newer
armband technologies which integrate the use of accelerometers with heat flux, skin
temperatures, and galvanic skin responses to estimate energy expenditure (Sense Wear,
Body Media, Pittsburgh, PA); however, findings regarding the precision of these tools are
conflicting. For example, one study (218) revealed that the mean estimated EEE with a
portable armband was approximately 117 kcal/d lower than that measured with DLW;
however, individuals comparisons between the armband and DLW were similar (218). In
another study (113) examining the validity of the Sense Wear Pro3 armband in estimating
EEE, this device was found to underestimate energy expenditure during running and
cycling at high exercise intensities.

New Technologies

Several new field technologies including mobile applications, online software,
and personal calorie tracking devices have been developed to estimate energy
expenditure in a field setting. For example, Sports Tracker, Calorie Counter, and Map
my Fitness are just a few of the mobile programs now available to assess one’s energy
status. Date regarding the validity and reliability of these programs in comparison to
direct calorimetry, indirect calorimetry, and or DLW is lacking. Future studies are needed
to address the precision of these new technologies.

*Dietary Energy Intake*

Energy balance represents the difference between total daily energy expenditure
and EI (EB=TEE-EI kcal/d) (55, 259) and, as noted above, EA which represents the
amount of EI remaining after exercise training for all other metabolic processes has been
operationally defined as EI minus EEE relative to kilograms of LBM (144). The
assessment of EI is thus essential for the calculation of energy balance and EA. Common
methods for assessing EI include diet records, food recalls, and food frequency
questionnaires (16). Prospective diet records may be kept for varying lengths of time. For
example, 3 to 16 day (234), 28 day (220), and even yearlong (227) diet records have been
used to assess EI. Day to day variability in EI has been reported to range from
approximately 2 to 37% in males and 1 to 33% in females (181, 227). Consequently, at
least 2 to 6 day diet records have been suggested to estimate EI with good accuracy
(181). Whether a longer period of dietary record collection improves the reported energy
intake appears to be less important than the participant’s characteristics such as body
weight and dietary restraint (159, 234).

Food recalls provide a retrospective record of dietary intake over a defined time
period which is usually 24 hours. Recalls can be administered with minimal cost and low
participant burden. To estimate the usual dietary intakes of participants, multiple recalls
on random, nonconsecutive days are recommended (16). Recalls may be obtained either
in person or by telephone administered interviews. Recalls by telephone interview have been shown to be practical, valid, and cost effective (80) and therefore are becoming an increasingly popular method of dietary assessment. Food frequency questionnaires are designed to obtain information about usual long-term food consumption patterns and are widely used in epidemiological studies (16). These questionnaires provide a rapid and cost effective way to estimate usual dietary intake. However, a greater percentage of under reporting has been documented using this method of dietary assessment when compared to 24 hour recalls (223).

A number of studies have noted the errors associated with using diet records to assess EI. Although EI will never be estimated without error (16), a number of strategies including the use of food models (25), photographs (146), nutritional training sessions (25), and weighing food and beverages (20) can be used to improve the accuracy of the self reported diet records. In addition, the collection of shorter 3 to 4 day instead of 7 day diet records has been shown to reduce participant burden and improve compliance (93).

A number of techniques have been used to validate diet records such as DLW (23, 131, 223, 234), 24 hour urine specimens with para-aminobenzoic acid tablets (223), and body weight in long term metabolic studies (160). These methods are not feasible for large scaled studies; therefore, an alternative to these aforementioned techniques is to test for low EI that are deemed to be physiologically impossible. An example of this includes the “Goldberg cut-off” (85) which is based on the concept that energy expenditure can be represented as REE multiplied by an activity factor. A ratio of EI to REE that is less than the activity factor is suggestive of under reporting. For example, if a PAL value of 1.85 is estimated for an exercising women whose reported EI and measured REE is 2000 and
1200 kilocalories/d, it is suggested that this woman is under reporting as the ratio of EI to REE is less than the 1.85 activity factor [2000/1200=1.67]. Several concerns regarding this cut-off have been reported in the literature (21-22): 1) investigators have selected overly conservatively small physical activity factors to not over-estimate the extent of under reporting, 2) the cut-off does not identify under reporters at the upper end of the distribution of energy intake and expenditure, and 3) the cut-off does not indentify over reporters (22). The sensitivity and specificity of the Goldberg cut-off can be improved with selecting with low, medium, or high activity factors that best match participants level of physical activity (22). Alternative approaches to accounting for under and over reporting of EI include using a cut-off based on predicted dietary reference intake prediction equations or a revised Goldberg cut-off that uses alternative REE equations that are more applicable to the participants being examined (159). Eliminating or adjusting for implausible reporters has been shown to impact EI and BMI associations (159). Future studies are needed to improve the sensitivity and specificity of methods to indentify implausible reporters.

What are the best tools to identify the presence of an energy deficiency and menstrual disturbance?

*Metabolic Markers*

In addition to the above methods, several metabolic markers including but not limited total triiodothyronine (T3) (58), ghrelin (1, 38, 58-59), leptin (48), insulin-like growth factor-1 (161), PYY (206), adiponectin (197), and C-reactive protein (197) have been used to assess energy status. It is important to note here that the term “energy
status” which will be used throughout this section implies an energy deficiency when conditions support EI being greater than TDEE and energy surplus when conditions support EI being greater than TDEE. The following discussion will be limited to T₃ and ghrelin which are the two metabolic markers measured for the purposes of this dissertation.

Peripheral thyroid metabolism is altered when energy intake and expenditure are mismatched and unaltered when energy balance is achieved, regardless of whether the deficit or surplus is created by energy intake or expenditure (53). In response to caloric restriction, diminished thyroid function exerts effects at the level of the hypothalamus, pituitary, and peripheral tissues. There’s conflicting literature as to whether thyroid stimulating hormone concentrations decrease in response to caloric restriction (53, 78). Type I monodeiodinase activities in the peripheral tissues are reduced leading to a concomitant reduction in thyroxine to T₃ conversion and thus T₃ concentrations (53, 78). These effects are related to the duration and extent of caloric restriction (53). Several studies have reported significant decreases in mean T₃ concentrations in response to an energy deficit (138, 144, 258) and lower T₃ concentrations in exercising women (58, 258) and exercising monkeys (257-258) with menstrual disturbances. In short term studies manipulating EI and EEE, significant reductions in T₃ have been observed below and EA of 30 kcal/kg LBM. More severe reduction in T₃ concentrations were also observed at an EA of 20 kcal/kg LBM (144). Significant perturbations in reproductive, other metabolic, and bone turnover makers have also been observed below an EA of 30 kcal/kg LBM. These findings suggest that EA could be used assess an energy deficiency
as lower T<sub>3</sub> concentrations have been observed in concomitant with lower EA values (103, 144).

In well controlled laboratory experiments, single measurements of T<sub>3</sub> in energy deficient participants (10 kcal/kg LBM) have been shown to fall within the range of energy balanced participants (45 kcal/kg LBM) (133). Loucks and colleagues (135) suggested that single measurements of metabolic hormones do not reliably identify energy deficient individuals as the normal ranges across the population are wide compared to the effects of energy deficiency on them (133). Caution must be exercised as measurements of individual T<sub>3</sub> may therefore not be the most precise method to assess participants energy and menstrual status.

Ghrelin is a peptide hormone which is produced in a distinct endocrine cell type of the stomach and gastrointestinal tract (54). It is also produced in the hypothalamus (91). In rodents, circulating ghrelin concentrations have been shown to increase during fasting and decrease when nutrients are sensed in the stomach (236). In humans, Fasting ghrelin concentrations have been shown to be negatively correlated with 24 hour EI (219). In particular, ghrelin concentrations decrease following EI with the decrease being related to the amount of calories ingested (127). Lower ghrelin concentrations have also been documented in obese than lean participants (237).

Several studies have reported higher ghrelin concentrations in exercising women with amenorrhea (58-59) and adolescent athletes with anorexia nervosa (1, 38). These elevated ghrelin concentrations may be related to the suppression of reproductive function in these amenorrheic women (205). In support, Vulliemoz and colleagues (244) showed that ghrelin infusion which increased plasma ghrelin levels almost 3 fold
significantly reduced LH pulsatility in female rhesus monkeys. These findings suggest that elevated ghrelin concentrations in exercising women may play a role in the suppression of reproductive function. Ghrelin may therefore prove to be a useful metabolic marker in assessing energy and menstrual status. No studies to date have however examined the relationship between EA defined as EI minus EEE and LBM and ghrelin concentrations.

Assessment of an Energy Deficiency and Menstrual Status

Although the above direct and indirect methods can be used to assess energy and menstrual status, no studies to date, to our knowledge, have examined the best method to predict an energy deficiency and menstrual disturbance. Moreover, no studies have examined this issue using a large sample of exercising women with varying menstrual status including ovulatory menstrual cycles, luteal phase defects, anovulatory menstrual cycles, oligomenorrhea, and amenorrhea. These are important questions to address as determining this best method, with the goal that it can be used in a field setting, will help to identify those exercising women at risk for the Female Athlete Triad. Early detection of an energy deficit will help clinicians, registered dieticians, mental health practitioners, coaches, athletic trainers, and or exercise physiologists to work with exercising women to prevent further negative health outcomes including menstrual disturbances and bone loss that occur secondary to an energy deficit.
Chapter 3

Changes in energy availability across the season in Division I female soccer players

Abstract

Low energy availability (EA) ((energy intake kcals - exercise kcals)/kgLBM) has been associated with menstrual disturbances and low bone mass. Few studies have examined the EA of athletes across a season. The purpose of this study was to assess the prevalence of and what contributes to low EA in Division I female soccer players across a season. Nineteen participants (18-21 years; VO$_{2\text{max}}$: 57.0±1.0 ml/kg/min) were studied during the pre, mid, and post season. Mean EA was overall lowest at mid and lower at mid than post season (31.5±3.7 vs. 42.7±3.4 kcal/kg LBM, p=0.004). Low EA (< 30 kcal/kgLBM) was observed in 9/19 (47.4%), 6/15 (40.0%), and 2/17 (11.8%) of participants during the pre, mid, and post season, respectively. Low EA was due to lower energy intake at lunch and dinner during pre season (p≤0.006) and breakfast and lunch during mid season (p=0.036). EA was inversely related to body dissatisfaction (r=-0.636, p=0.014) and drive for thinness (r=-0.564, p=0.036) during mid season. Although the majority of Division I female soccer players are not at risk for low EA, a concerning proportion exhibited low EA levels at pre or mid season. Further studies should explore strategies to prevent and monitor low EA in these athletes.
Introduction

The Female Athlete Triad is a syndrome of three interrelated conditions: low energy availability (EA), amenorrhea, and osteoporosis. These conditions, either alone or in combination, pose significant health risks to exercising females (171). EA is the amount of dietary energy intake remaining after exercise training for all other metabolic processes (EI-EEE/kg LBM) and laboratory studies indicate that negative metabolic, reproductive, and bone related changes occur when EA drops below 30 kcal/kg LBM (103, 144). Athletes at the greatest risk for low EA are those who restrict dietary energy intake (EI), exercise for long periods of time, and limit their food choices (171). The game of soccer is characterized by intermittent, high intensity exercise that necessitates the use of both the non-oxidative and oxidative energy systems. The training demands placed on female soccer players change across the season (213); it is therefore important to characterize the energy status of these athletes during each of the training phases to determine their risk of low EA and consequently the Triad.

To date, no studies have examined the risk of low EA in Division I female soccer players or changes in EA across a competitive season in any sport. Low EI ranging from 1750 to 2880 kcal/d (40) relative to a total energy expenditure on average of 2218 kcal/d (79) and high exercise energy expenditure (EEE) (213), suggest that female soccer players may be at risk of low EA. Similar results were observed in male professional soccer players who reported an average 24 hour energy deficit of 419 kcal/d during the competitive season (74). These findings suggest that Division I female soccer players may be at risk of low EA during the pre and competitive season.
Disordered eating attitudes may predispose female athletes to lower EI. High drive for thinness (DT) and dietary cognitive restraint are common eating attitudes of individuals at risk for disordered eating and have been observed in energy deficient exercising premenopausal women (84, 196). An unexpected high prevalence of eating disorders (28%) was observed among female athletes participating in ball-game sports (224). Eating behaviors such as EI of particular meals and number of eating occasions have also been shown to impact total EI (92, 233). Eating a number of small meals and snacks daily may be a practical strategy that allows for increased EI while reducing the gastrointestinal discomfort of infrequent large meals (92). Alternatively, a reduced number of eating occasions and/or low EI at particular meals may contribute to low EA in athletes. Taken together, negative eating attitudes, lower EI at particular meals, and/or lower number of eating occasions may therefore be associated with lower EI and consequently low EA in Division I female athletes.

The purpose of this study was to test the following hypotheses: 1) levels of EA will be lower during the pre and mid season when compared to the post season, 2) lower EA will be due to lower EI, 3) lower EA will be associated with negative eating attitudes, and 4) lower EI will be due to a combination of lower EI of particular meals and number of eating occasions.

Methods

Study design

During the pre (3 consecutive days in August), mid (3 consecutive days in October), and post (3 consecutive days in November) season, repeated measures of EA,
EI, EEE, body composition, aerobic fitness, metabolism, eating attitudes, and demographics were obtained.

Participants

Members of a NCAA Division I women’s soccer team at a university in the northeastern United States participated in this study. During an initial visit, study details and participation requirements were explained, and written informed consent was obtained. The study was approved by the university’s Institutional Review Board for Research with Human Participants. To be included in the study, participants had to be current members of a Division I women’s soccer team. Twenty-five participants signed the informed consent. Two participants later withdrew due to time commitment, two from lack of interest, and two due to injuries. A total 19 participants completed the study.

Energy availability

EA was operationally defined as EI minus EEE relative to kilograms of lean body mass (kcal/kg LBM) (144) and calculated during the pre, mid, and post season. Measures of EI and EEE were performed for the same 3 consecutive days during the pre, mid, and post season. Measures of LBM were obtained during one of the 3 consecutive days during all time points. EA was assessed on typical training days during the pre (3 practice days), mid (2 practice and 1 home game day), and post season (3 non practice/game days).

Dietary energy intake

EI was assessed from 3-day diet logs during the pre, mid, and post season. A member of the research team with graduate training in sports nutrition instructed participants how to accurately record all foods and beverages consumed and provided
participants with a food amounts packet. The packet contained diagrams illustrating container sizes, cuts of meat, and various circles and squares which are used when estimating portion sizes for foods like bowls of cereal. Participants were encouraged to use these scaled diagrams as a guide for describing dimensions and sizes. Also included in the packet was a sample page of an accurately completed diet record provided as a reference. Participants were asked to record all food and beverages consumed for 3 consecutive days, including time of day, location, and meal type i.e. breakfast. The 3-day diet logs were reviewed for completeness and accuracy when returned. Nutrient data from the 3-day logs were coded and analyzed using the Nutritionist Pro Diet Analysis software (Axxya Systems, Stafford, TX software version 4.5). The average EI from the 3-day diet logs provided the EI to compute EA during the pre, mid, and post season.

**Exercise energy expenditure**

Three methods were used to capture EEE: 1) Polar Team² software, 2) Polar FT4 heart rate monitors, and 3) purposeful exercise logs. First, energy expended during team training sessions such as soccer practice, soccer games, and weight lifting was measured using the OwnCal feature of the Polar Team² software (Polar Electro Oy, Kempele, Finland) (49). Second, energy expended during non team training sessions were also measured using the OwnCal feature of the Polar FT4 heart rate monitor. The OwnCal feature has been validated for the use in calculating EEE from heart rate (49). Third, for the few (<10%) purposeful exercise sessions in which participants were unable to wear the Polar heart rate monitors, the compendium of physical activities was used to determine the appropriate metabolic equivalent (MET) level for the exercise performed (6). Participants recorded the duration, mode, and intensity of all purposeful exercise
sessions on physical activity logs for 3 consecutive days during the pre, mid, and post season. This information was used to select the appropriate MET level. To calculate the energy expended during these exercise sessions, the following equation was used:

\[
EEE = \text{duration (minutes)} \times \left( \frac{\text{METs} \times 3.5 \times \text{weight (kg)}}{200} \right)
\]

Anthropometrics

Total body weight was measured by a digital scale in the laboratory to the nearest 0.01 kg wearing t-shirt and gym shorts after an overnight fast between 0700 and 1000 during the pre, mid, and post season. Height was measured to the nearest 1.0 cm without shoes during the pre season. Body mass index (BMI) was calculated as a ratio of weight to height (kg/m\(^2\)). Body composition, including percent body fat, fat mass, and LBM was analyzed during the pre, mid, and post season by a certified technician using dual-energy x-ray absorptiometry (DXA). The participants were scanned on a GE Lunar iDXA scanner (General Electric Lunar Corporation, Madison, WI, enCORE 2008 software version 12.10.113).

Eating attitudes questionnaire

To assess eating attitudes, the Eating Disorder Inventory (EDI-2) which measures 11 subscales of attitudes and behaviors related to disordered eating was administered to all participants during the mid and post season (82). Data as reported were limited to 3 subscales, drive for thinness (DT), bulimia, and body dissatisfaction (BD) that have been related in prior studies to chronic energy deficiency in female athletes (42, 84, 196).

Eating behaviors

The EI of each eating occasion represented all the food and beverages participants listed under each eating occasion. All food and beverages consumed within a 30 minute
period in the same location were included in the same eating occasion (34). The EI of same eating occasions from each day of the 3-day diet logs were averaged together to provide the average EI of each eating occasion during the pre, mid, and post season. Only the average EI of breakfast, lunch, and dinner are reported due to the small sample size of the other eating occasions. The total number of eating occasions reported for each day of the 3-day diet logs were averaged together to provide the average number of eating occasions during the pre, mid, and post season.

**Exercise testing**

Measurement of maximal aerobic capacity (VO$_{2\text{max}}$) was performed during the pre and post season on a treadmill using indirect calorimetry and the modified Åstrand protocol (9). Gas exchange was continuously monitored by a breath-by-breath system (SensorMedics Vmax metabolic cart, Yorba Linda, Calif., USA). VO$_{2\text{max}}$ was achieved if 3 of the 4 following criteria were obtained: (1) attainment of age-predicted maximal heart rate (208-(0.7*age)); (2) respiratory exchange ratio $\geq$ 1.1; (3) plateau in oxygen consumption despite an increase in exercise workload; (4) attainment of a rating of perceived exercise score $\geq$18.

**Metabolic blood sample**

Blood was collected after an overnight fast between 0700 and 1000 during the pre, mid, and post season. Participants were asked to lie in the supine position for at least 15 minutes after which a General Clinical Research Centre nurse obtained a blood sample via venipuncture. Samples were allowed to clot for at least 30 minutes at room temperature and then spun in a centrifuge where after serum was stored at -80$^\circ$ Celsius until analysis. Serum samples were assayed in duplicate for total triiodothyronine (T$_3$)
using an Immulite chemiluminescent assay (First Generation Immulite 1000, Siemens, Deerfield, IL). The sensitivity of the assay was 35 ng/dL and the intraassay and interassay coefficients of variation were 10.3% and 13.3%, respectively.

**Statistical analysis**

All variables were tested for outliers and normality using box plot analyses and Shapiro-Wilk test of normality, respectively. Extreme outliers were not included in our analyses. Paired t-tests were performed when only two time points were available. Pearson’s correlation coefficient analyses were performed for variables of interest. Repeated measures analysis of variance (ANOVA) was performed to examine the changes across the pre, mid, and post season. Post hoc testing to reveal when significant time effects occurred was performed using t-tests with a Bonferroni correction, where \( p < 0.017 \) was considered significant. Independent t-tests were used to compare differences between groups. When variables were not normally distributed, Friedman Tests were performed to examine changes across the pre, mid, and post season. Kruskal-Wallis tests were used to compare differences between groups. The Wilcoxon rank sum test was then used to reveal when significant time effects occurred. Data are reported as means ± SEM, and \( p \leq 0.05 \) was considered statistically significant. All data were analyzed using SPSS for Windows (version 18; Chicago, Ill., USA).

**Results**

**Participant characteristics and demographics**

Descriptive data for all participants during the pre, mid, and post season are shown in Table 2-1. No differences in weight, BMI, \( \text{VO}_{2\text{max}} \), percent body fat, fat mass,
and LBM were detected across the season ($p>0.05$). Participants exercised an average of 135 ± 3, 101 ± 1, and 18 ± 1 minutes/day during the pre, mid, and post season, respectively. Average exercise heart rates were 131 ± 2 and 132 ± 2 bpm during the pre and mid season, respectively.

Table 2-1. Descriptive characteristics and anthropometrics of Division I female soccer players across the season (n=19)

<table>
<thead>
<tr>
<th></th>
<th>Pre Season</th>
<th>Mid Season</th>
<th>Post Season</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>19.23 ± 0.3</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Height (cm)</td>
<td>165.6 ± 1.2</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>60.6 ± 1.4</td>
<td>61.3 ± 1.4</td>
<td>61.0 ± 1.4</td>
<td>p = 0.146</td>
</tr>
<tr>
<td>BMI ($\text{kg/m}^2$)</td>
<td>22.2 ± 0.3</td>
<td>22.3 ± 0.3</td>
<td>22.2 ± 0.3</td>
<td>p = 0.165</td>
</tr>
<tr>
<td>VO$_2$ max (ml/kg/min)</td>
<td>57.0 ± 1.0</td>
<td>-</td>
<td>56.8 ± 1.3</td>
<td>p = 0.344</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>22.5 ± 1.1</td>
<td>22.9 ± 1.1</td>
<td>22.6 ± 1.1</td>
<td>p = 0.253</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>13.2 ± 0.9</td>
<td>13.6 ± 0.9</td>
<td>13.3 ± 0.9</td>
<td>p = 0.162</td>
</tr>
<tr>
<td>Lean body mass (kg)</td>
<td>44.6 ± 0.7</td>
<td>44.9 ± 0.7</td>
<td>44.9 ± 0.7</td>
<td>p = 0.376</td>
</tr>
</tbody>
</table>

Values are shown as mean ± SEM.

**Energy availability**

EA of individual female soccer players in presented in Figure 2-1. Low EA (<30 kcal/kg LBM) was observed in 9 of 19 (47%) during the pre season, in 6 of 15 (40%) during the mid season, and in 2 of 17 (12%) of participants during the post season. Five of 19 (26%) participants demonstrated low EA during both the pre and mid season. EA
and its components are presented in Figure 2-2. Repeated measures ANOVA revealed a curvilinear change in EA over time (time effect $F = 11.6$, $p = 0.017$) demonstrating a lower mid than post season EA ($p = 0.04$) (Figure 2-2a). EI decreased over time (time effect $F = 9.844$, $p = 0.007$). EI was lower mid ($p = 0.008$) and post ($p = 0.022$) compared to pre season (Figure 2-2b). EEE decreased over time (time effect $F = 20.945$, $p < 0.001$) demonstrating a lower mid ($p = 0.024$) and post ($p = 0.001$) than pre season EEE and lower post ($p = 0.26$) than mid season EEE (Figure 2-2c). No significant changes in LBM were observed across the season ($p = 0.376$) (Figure 2-2d).

Figure 2-1. Energy availability of individual Division I female soccer players during the pre, mid, and post season. Open circles represent EA of individual female soccer players. Filled circles represent EA of individual female soccer players with low EA (<30 kcal/kg LBM) during the pre and mid season. Solid bars represent the mean EA (kcal/kg LBM). Dashed line represents a threshold of EA (30 kcal/kg LBM) which has previously been associated with negative reproductive, metabolic, and bone health outcomes in laboratory studies.
Figure 2-2. Energy availability (a), dietary energy intake (b), exercise energy expenditure (c), and lean body mass (d) of Division I female soccer players during the pre, mid, and post season. Data are expressed as mean ± SEM. \(^a P < 0.05\) versus values at pre season; \(^b P < 0.05\) versus values at mid season; \(^c P < 0.05\) versus values at post season.
Eating attitudes

Scores of eating attitudes are presented in Table 2-2. No differences in BD, DT, or bulimia scores were observed between the mid and post season (p>0.05). Higher BD (5.8 ± 1.5 vs. 1.3 ± 0.6; Kruskal Wallis Test, p = 0.007) was detected in participants with low compared to higher EA during the mid season. When all participants are combined, a negative relationship was observed between EA and BD (r = -0.636, p = 0.014) and DT (r = -0.564, p = 0.036) during the mid season.

<table>
<thead>
<tr>
<th></th>
<th>Mid Season mean±SEM</th>
<th>Range</th>
<th>Post Season mean±SEM</th>
<th>Range</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body dissatisfaction (BD)</td>
<td>2.9 ± 0.8</td>
<td>0-12</td>
<td>2.1 ± 0.5</td>
<td>0-7</td>
<td>p = 0.209</td>
</tr>
<tr>
<td>Interoceptive awareness</td>
<td>1.5 ± 0.5</td>
<td>0-6</td>
<td>0.6 ± 0.2</td>
<td>0-4</td>
<td>p = 0.036*</td>
</tr>
<tr>
<td>Drive for thinness (DT)</td>
<td>2.8 ± 1.1</td>
<td>0-16</td>
<td>1.1 ± 0.4</td>
<td>0-6</td>
<td>p = 0.116</td>
</tr>
<tr>
<td>Bulimia</td>
<td>1.1 ± 0.3</td>
<td>0-4</td>
<td>0.9 ± 0.3</td>
<td>0-4</td>
<td>p = 0.167</td>
</tr>
<tr>
<td>Ineffectiveness</td>
<td>0.6 ± 0.3</td>
<td>0-4</td>
<td>0.3 ± 0.2</td>
<td>0-3</td>
<td>p = 0.058</td>
</tr>
<tr>
<td>Interpersonal distrust</td>
<td>1.3 ± 0.5</td>
<td>0-8</td>
<td>1.2 ± 0.4</td>
<td>0-6</td>
<td>p = 0.750</td>
</tr>
<tr>
<td>Perfectionism</td>
<td>7.8 ± 0.9</td>
<td>1-17</td>
<td>7.4 ± 1.0</td>
<td>2-17</td>
<td>p = 0.477</td>
</tr>
<tr>
<td>Maturity fears</td>
<td>4.1 ± 0.8</td>
<td>1-13</td>
<td>3.6 ± 0.8</td>
<td>0-13</td>
<td>p = 0.776</td>
</tr>
<tr>
<td>Asceticism</td>
<td>2.6 ± 0.6</td>
<td>0-10</td>
<td>2.7 ± 0.4</td>
<td>0-8</td>
<td>p = 0.633</td>
</tr>
<tr>
<td>Impulse regulation</td>
<td>0.5 ± 0.3</td>
<td>0-4</td>
<td>1.4 ± 0.6</td>
<td>0-10</td>
<td>p = 0.084</td>
</tr>
<tr>
<td>Social security</td>
<td>1.9 ± 0.4</td>
<td>0-6</td>
<td>1.6 ± 0.4</td>
<td>0-6</td>
<td>p = 0.318</td>
</tr>
</tbody>
</table>

*, p < 0.05 vs. mid season mean value.
Eating behaviors

EI of the three main eating occasions i.e., breakfast, lunch, and dinner is presented in Figure 2-3. A lower EI at lunch (365 ± 70 vs. 1006 ± 82 kcal/d; p < 0.001) and dinner (646 ± 38 vs. 990 ± 98 kcal/d; p = 0.006) was detected in participants with low compared to higher EA during the pre season (Figure 2-3a). A lower EI at breakfast (280.4 ± 32.9 vs. 418.5 ± 44.0 kcal/d; p = 0.036) and lunch (450.6 ± 74.6 vs. 816.3 ± 89.8 kcal/d; p = 0.013) was observed in participants with low compared to higher EA during the mid season (Figure 2-3b). Due to the small number of participants (n=2) with low EA during the post season, statistical analyses regarding differences in EI of particular eating occasions between participants above and below an EA of 30 kcal/kg LBM were not performed. No differences in the number of eating occasions were observed between participants with low and higher EA during the pre, mid, or post season (p>0.05). Participants exhibited an average of 5.0 ± 0.3, 4.5 ± 0.4, and 3.7 ± 0.1 eating occasions during the pre, mid, and post season, respectively.
Figure 2-1. Energy intake of eating occasions during the pre (a) and mid (b) season of Division I female soccer players. Filled bars represent higher energy availability (≥ 30 kcal/kg LBM). Open bars represent lower energy availability (< 30 kcal/kg LBM). Data are expressed as mean ± SEM. *P < 0.05.
Total triiodothyronine

Repeated measures ANOVA revealed no change in T$_3$ concentrations across the season (113.9 ± 4.2 pre, 117.3 ± 4.2 mid, 117.8 ± 5.5 post ng/dL; time effect F = 0.163, p = 0.851). In addition, no differences in T$_3$ concentrations were observed between participants with low and higher EA during the pre, mid, or post season (p>0.05).

Discussion

This study was the first to examine the risk of low EA (<30 kcal/kg LBM) (EI-EEE/kg LBM), in Division I female soccer players at several time points across the season. EA declined by 19% from pre to mid season and then improved by 35% at post season. During all time points, the mean EA of our soccer players remained above an EA threshold of 30 kcal/kg LBM previously associated with negative metabolic, reproductive, and bone health outcomes (103, 144). However, a concerning proportion (40%) experienced low EA at the pre and or mid season time point. The increase in EA from mid to post season was attributable to decreased EEE. We observed a negative association between BD and EA, DT, and EA, and higher BD in the female athletes with low EA. Lastly, we determined that specific meals, such as lunch, could be pinpointed as main contributors to low EA. These results add to the small but growing number of findings from studies attempting to determine EA of female athletes in a field setting (62, 98, 196, 204).

An important contribution of the current study is that the majority of Division I female soccer players are not at risk of low EA; however, a concerning percentage exhibited low EA during the pre (47.4%) and mid (40.0%) season. Of the 5 participants
who exhibited low EA during the pre and post season, higher EA (≥ 30 kcal/kg LBM) was observed in 4 of these participants during the post season indicating that the risk of low EA might be seasonal and thus reversible in Division I female soccer players. Findings from recent cross-sectional studies (134, 196, 204) in free-living female athletes revealed lower mean EA values compared to those obtained in the present study (38.9 pre, 31.5 mid, and 42.7 post; kcal/kg LBM). In high school athletes in a range of sports, Hoch et al. 2009 found that 6% of athletes presented with low EA (<30 kcal/kg LBM). The prevalence of low EA in the current study ranged from 12 to 47%, and thus represents a greater prevalence than that reported by Hoch et al. 2009. This finding might be explained by differences in EEE as the high school participants of Hoch et al. 2009 exercised 57 minutes/d while the collegiate soccer players in the current study exercised an average of 135 and 101 minutes/d during the pre and mid season, respectively.

The biggest contributor to low EA in the present study was low EI. Findings from several studies have showed that negative eating attitudes are associated with lower EI. For example, higher DT, bulimia, and BD scores have been observed in energy deficient exercising females with severe menstrual disturbances (42, 196). These findings are similar to the results of the present study as we observed a negative association between BD and EA, DT and EA, and higher BD in the female athletes with low EA. Thus, these results provide a corroborate measure for the low EA observed and a plausible explanation for the low EA observed in some of the athletes in the present study.

Findings from recent studies examining the coupling between EI and EEE suggest that inadvertent under-eating leads to low EI. The lack of an increase in EI in response to
an acute exercise induced energy expenditure i.e., inadequate compensation has been observed in young, obese, and non-obese men and women (108, 221, 254). The above studies suggest that the lack of significant difference in EI between the mid and post season despite greater EEE during the mid season in our soccer players could be due to inadvertent dietary compensation to match EEE.

Lower EI at lunch and dinner during the pre season as well as breakfast and lunch during the mid season were observed in the athletes who presented with low EA. Our results are similar to those of Tomten and Hostmark (2006) who reported lower EI at dinner in energy deficient runners with irregular menstrual function. During the pre season of the current study, foods and beverages for breakfast were provided ad libitum in a team meeting room, athletes were responsible for preparing or purchasing their lunch, and a catered dinner was provided each night after team training sessions. During the mid season, athletes were responsible for all of their meals, except for dinner following the home soccer game. The lower EI in the athletes with low EA was found in part, at meals in which athletes were responsible for preparing or purchasing their own food and beverages i.e., pre season lunch, and mid season breakfast and lunch. It is possible that participants with a propensity to exhibit low EA will be less willing to seek out food when the responsibility is theirs. Lower EI was also observed in the low EA group at pre season dinner when catered meals were provided. It is possible that moderate intensity exercise training (mean heart rate: 131.2 ± 1.7 beats per minute) immediately prior to dinner temporarily decreased hunger (152) and therefore negatively impacted EA, and that participants with a propensity to develop low EA are more susceptible to this appetite suppression. Perhaps nutritional strategies aimed at increasing
EI during meals, in particular lunch, or eating meals together as a team either before or with sufficient time after exercise training might prevent the low EA observed in some of these athletes.

During short term manipulations of EI and EEE, Loucks and Thuma (2003) observed significant reductions in $T_3$ concentrations below an EA of 30 kcal/kg LBM in regularly menstruating, previously sedentary females. These findings are similar to those of the present study as changes in $T_3$ concentrations were not observed in Division I female soccer players who exhibited mean EA levels greater than 30 kcal/kg LBM during the pre, mid, and post season. Lower $T_3$ concentrations were not observed in the players who exhibited low EA at some point during the season. Exercise training has been shown to have a stimulatory effect on $T_3$ production in adult male mice (104) and maintain $T_4$ secretion and $T_3$ synthesis during exercise-induced weight loss in female Sprague-Dawley rats (105). Although low EA was achieved in some participants, $T_3$ concentrations could have been preserved in the presence of chronic exercise training in these female soccer players as suggested by the above findings of Katzeff and colleagues (1988, 1991).

To date, no studies have examined how well changes in EA correspond to changes in body weight or energy balance (EB). The current study did not examine 24 hour EB or its components. Reductions in resting metabolic rate have been documented in female athletes with menstrual disturbances (58). Moreover, increases and decreases in non-exercise associated activity thermogenesis have been observed in a state of positive and negative EB, respectively (129). Changes in these parameters of EB along with training adaptations such as increases in plasma volume (46) and body water stores which
accompany glycogen storage (179) could have contributed to the lack of changes in body weight observed, despite changes in EA in our soccer players.

Limitations of this study include the fact that EI was collected using self-report 3-day diet logs. Several studies have shown inaccuracy when measuring EI using self-reported food records, particularly related to underreporting (203). However, all of the participants were trained to accurately record food intake and provided with both a foods amount packet and completed diet record as a reference. Another limitation is the lack of a larger sample size and thus low power to detect differences between groups with low versus higher EA.

**Conclusion**

In this first study to examine the risk of low in EA in free-living elite female athletes across a competitive season, we observed that a concerning percentage of these athletes exhibited low EA at some point during the season. Negative eating attitudes i.e., high BD were observed in the athletes with low compared to higher EA. We found no changes in T3 concentrations across the season in female soccer players whose mean EA remained above an EA threshold which has been previously associated with negative health outcomes. Changes in EA in Division I female soccer players appear to be reversible and seasonal as few participants showed low EA during the post season. Nutritional strategies for Division I female soccer players should focus on monitoring eating attitudes and increasing EI at meals, particularly lunch, to prevent the low EA observed during the pre and mid season.
Chapter 4

Energy availability assessed with diet logs, exercise logs, and heart rate monitors does not discriminate menstrual status in exercising women

Introduction

Approximately half of exercising women experience subtle menstrual disturbances such as luteal phase defects and anovulation, and one third of exercising women may be amenorrheic (64). Lower bone mineral density (66), stress fractures (266), higher injury rates (182), disordered eating (84, 241), altered vascular function (177), reductions in resting metabolic rate (124, 168), and suppressed metabolic hormone concentrations (58) have been documented in women with exercise associated menstrual disturbances (EAMD). Prospective exercise training studies show that low energy availability (EA) is causally related to menstrual disturbances in exercising women (32, 193, 256-257, 260).

EA has been operationally defined by one investigator (144) as dietary energy intake (EI) minus exercise energy expenditure (EEE) relative to kilograms of lean body mass (LBM) i.e., (EA = EI-EEE/kg LBM). This variable represents the amount of EI remaining after exercise training for all other metabolic processes such as reproduction, thermoregulation, cellular maintenance, locomotion, and growth (134, 246). Conditions of low EA (<30 kcal/kg LBM) have been associated with reduced levels of metabolic hormones (97, 136, 138, 144) and unfavorable alterations in bone markers (103). In addition, reductions in luteinizing hormone (LH) pulsatility have been observed below an
EA of 30 kcal/kg LBM in previously sedentary women during short-term manipulations of EI and EEE in a controlled laboratory setting (144). Although these studies (97, 103, 136, 138, 144) define a critical EA threshold of 30 kcal/kg LBM) for the initiation of reduced LH pulsatility, few investigators have examined whether an EA of 30 kcal/kg LBM discriminates normal ovarian function from either subtle (luteal phase defects or anovulation) or severe (oligomenorrhea or amenorrhea) menstrual disturbances in exercising women. The most severe reductions in LH pulsatility have been observed in amenorrheic runners (142, 240). Less severe reductions in LH pulsatility have been observed in exercising women with eumenorrhea (50), anovulatory menstrual cycles (187), and oligomenorrhea (240). Severe reductions in EA below 30 kcal/kg LBM have been shown to produce incremental reductions in metabolic hormones, such as total triiodothyronine and suppression of bone formation markers (103, 144). It is unclear if an EA of 30 kcal/kg LBM or lower is associated with a dose response relationship with menstrual disturbances of increasing severity progressing from luteal phase defects and anovulatory menstrual cycles to oligomenorrhea, and amenorrhea (68).

In order to produce a defined level of EA, calculated as EI minus EEE relative to kilogram of LBM, Loucks et al. (103, 144) controlled EI with a clinical dietary product (Ensure Plus, Ross Laboratories, Columbus, OH) and used indirect calorimetry to monitor the target level of EEE. These methods to estimate EI and EEE are not feasible or practical for most free living exercising women. In the few studies (98, 134, 196, 204) that have examined EA in free living exercising women, EA has been defined inconsistently and measured using a variety of methods. Accelerometers (62), indirect calorimeters (204), and physical activity logs analyzed with the Ainsworth compendium
(5-6) have been used to estimate EEE. Likewise, a number of methods such as dual energy x-ray absorptiometry (DXA) (115, 121, 196, 204), underwater weighing (170), bioelectrical impedance (118) and skin fold measurements (62) have been used to estimate body composition. Most studies have used self reported diet logs for various lengths of time to estimate EI (62, 98, 115, 196, 233, 261). Due to the small sample sizes and inconsistencies in the methods used in the above mentioned studies (62, 98, 134, 204), the role of low EA as a predictor of menstrual abnormalities remains unknown. Conventional methods available to most exercising women such as diet logs, exercise logs, and or heart rate monitors to estimate EA, defined as EI minus EEE relative to LBM (144), should be used to test the role of EA in predicting menstrual status in a large population of free living exercising women.

The purpose of current study was to examine whether EA when assessed with conventional methodologies i.e. self reported diet logs, exercise logs, and heart rate monitoring discriminates disruptions in ovarian function in premenopausal exercising women. We hypothesized the following: 1) an EA of 30 kcal/kg LBM will discriminate ovarian function (ovulatory vs. exercise associated menstrual disturbances which include luteal phase defects, anovulatory menstrual cycles, oligomenorrhea, and amenorrhea) such that a significantly higher frequency of menstrual disturbances will be observed below an EA of 30 kcal/kg LBM when compared to the frequency of disturbances observed above an EA of 30 kcal/kg LBM, and 2) below an EA of 30 kcal/kg LBM, menstrual cycle disturbances will progress in severity from luteal phase defects to anovulatory menstrual cycles to oligomenorrhea, and finally amenorrhea as EA becomes incrementally lower.
Methods

Experimental Design

We combined data from two ongoing studies performed at the University of Toronto and The Pennsylvania State University. Both studies were designed to examine menstrual disturbances and alterations in energy balance in premenopausal women. The current study includes data from 100 exercising women in whom assessments of menstrual status and EA were performed. Measurements of the variables of interest, i.e., menstrual status, body composition, EI, EEE, and demographics were conducted by the same investigators at both sites using similar methods. To compare the usefulness of EA assessed with conventional methods to discriminate menstrual status to the association of other measures of energy status with menstrual status, we assessed fasting total triiodothyronine concentrations, resting energy expenditure (REE), and ratio of actual to predicted REE (REE/pREE), a marker of energy status in our lab papers (66, 84, 206).

Participants

Ninety-one participants were recruited by flyers posted on campus and in the surrounding community, newspaper advertisements, and classroom announcements targeting exercising women for a study on women’s health. Initial eligibility criteria included: 1) no history of or current serious medical conditions, 2) no current clinical diagnosis of an eating or psychiatric disorder based on self-report or an interview with a clinical psychologist or licensed clinical social worker, 3) age 18-35 years, 4) non-smoking, 5) no medication use that would alter metabolic or reproductive hormone concentrations, 6) ≥ 2 hrs/wk of purposeful exercise; 7) no history of or clinical diagnosis of polycystic ovarian syndrome (PCOS) and/or a free androgen index (FAI), calculated as
(total testosterone (nmol/L) / sex hormone binding globulin (SHBG) (nmol/L)) * 100) (200), > 6 in participants in which these hormones were measured. An FAI greater than 6.0 has been reported to be consistent with hyperandrogenemia (88, 198) and represents values greater than three standard deviations in our reference population (n=37) which consisted of healthy premenopausal exercising women (18-35 years) with documented ovulatory menstrual status (61, 64).

Screening Procedures

During an initial visit, participants were informed of the purpose, procedures, and potential risks of participation in the study before signing an informed consent approved by either the Human Ethics Board at the University of Toronto or the Institutional Review Board at The Pennsylvania State University. Once consent was obtained, height and weight were measured, and participants completed questionnaires to assess demographics, medical history, exercise history, menstrual history, eating behaviors, bone health, and mental health. A physical exam was performed on most participants by an on-site clinician to determine overall health and check for physical symptoms of PCOS such as acne or hirsutism. In addition, in most participants a fasting blood draw was analyzed for a complete blood count, basic chemistry panel, and an endocrine panel which included measures of LH, follicle stimulating hormone, thyroid stimulating hormone, thyroxine, prolactin, dihydroepiandrosterone (Quest Diagnostics, Pittsburgh, PA), total testosterone, and SHBG to rule out illness or endocrine or metabolic disease for most participants. Participants met with a General Clinical Research Center (GCRC) registered dietitian or trained laboratory personnel to receive instructions for completion of 3-day diet logs (2 weekdays and 1 weekend day). Additionally, dual-energy x-ray
absorptiometry (DXA) scans of the total body, lumbar spine, and dual femur were performed to assess bone mineral density (BMD) and body composition.

**Aerobic Capacity**

Peak aerobic capacity (VO$_2$ peak) was measured on a treadmill by indirect calorimetry using an on-line MedGraphics Modular VO$_2$ System (St Paul, MN) or SensorMedics Vmax metabolic cart (Yorba Linda, Calif., USA) during baseline using methods that have previously been published (58).

**Resting Energy Expenditure**

Resting energy expenditure was measured by indirect calorimetry using a Sensormedics Vmax metabolic cart (Yorba Linda, CA) using methods that have previously been published (196). We compared laboratory assessed REE with a predicted REE (pREE) using the Harris-Benedict equation to estimate how much each individual’s measured REE deviated from the pREE. We operationally defined energy deficiency as a REE:pREE ratio less than 0.90 (66, 84, 206). We choose to use this as our operational definition of energy deficiency to discriminate the exercising women who may present with an energy deficiency from those who are energy replete (84).

**Anthropometrics**

Total body weight was measured by a digital scale each week for 4 weeks in the laboratory to the nearest 0.01 kg wearing t-shirt and gym shorts. The mean of these measurements in presented. BMI was calculated as a ratio of weight to height (kg/m$^2$). Height was measured to the nearest 1.0 cm without shoes. Body composition, including percent body fat, fat mass (FM), fat free mass (FFM), and LBM was analyzed by a certified technician using DXA on one of three machines. The majority of participants
were scanned on either a GE Lunar Prodigy DXA scanner (n=57) (GE Lunar Corporation, Madison, WI, enCORE 2002 software, version 6.50.069) or a GE Lunar iDXA scanner (n=26) (General Electric Lunar Corporation, Madison, WI, enCORE 2008 software version 12.10.113). Remaining participants were scanned on a Hologic QDR4500 DXA scanner (n=8) (Hologic Inc., Bedford, MA). Consistent with the International Society of Clinical Densitometry guidelines, cross calibration studies were performed to remove systematic bias between the systems. For the cross calibration study between the Lunar Prodigy and Lunar iDXA, fourteen participants were scanned in triplicate on both machines. The majority (n=8) were scanned on both machines within 5 days while approximately one month lapsed between scans for some participants (n=6). The values for body composition obtained on each scanner were found to be highly correlated (r=0.97 BF%, r=0.99 FM, and r=0.93 LBM) with no significant difference between the population mean values. For the cross calibration study between the Hologic QDR4500W and the Lunar iDXA, thirty-two participants were scanned in duplicate on both machines on the same day. Equations were derived using simple linear regression to remove biases, and body composition values obtained from both the Lunar Prodigy and the Hologic QDR-4500W were calibrated to the Lunar iDXA.

**Menstrual Status**

The classification of menstrual status was based on self-reported menstrual histories, menstrual calendars used to chart menstrual symptoms i.e., cramps, bleeding, spotting, discharge, etc., and daily measurements of urinary estrone-1-glucuronide (E1G), pregnanediol glucuronide (PdG), and mid-cycle LH profiles. Participants who self-reported eumenorrheic menstrual status, defined as regular menstrual cycle intervals of
26-35 days (62, 64, 68), were monitored for two to three menstrual cycles. Participants who self-reported no menses within the past 3 months or 6 or fewer menses within the past year collected first morning urine samples beginning on an arbitrary day for 28 days.

Ovulatory status was confirmed by the presence of a urinary LH peak, identified as a peak concentration above 25 mIU/ml occurring after a mid-cycle E1G peak greater than 35 ng/ml, and followed by a peak luteal phase PdG concentration above 5 µg/ml in participants who exhibited menstrual cycles of 26-35 days (64). Luteal phase defects were confirmed when the luteal phase was either less than 10 days (short) or when the sum of the 3 day mid luteal peak PdG (sum of mid luteal peak PdG ± 1 day) was less than 10 µg/ml and when the PdG peak concentration was below 5 µg/ml but greater than 2.5 µg/ml in participants who exhibited menstrual cycles of 26-35 days (inadequate) (64). Anovulatory cycles were confirmed as cycles in which a minimal increase in E1G was observed concomitantly with a failure of LH to rise at midcycle and when a luteal phase exhibited no increase in PdG concentration above 2.5 µg/ml in participants who exhibited menstrual cycles of 26-35 days (64). Oligomenorrhea was confirmed if menses occurred at intervals of 36-90 days and if participants self-reported 6 or less menstrual cycles in the last year prior to the study. Lastly, functional hypothalamic amenorrhea was assessed by confirming a negative pregnancy test, no menses in the past 90 days, and chronically suppressed E1G and PdG profiles (64).

After consideration of menstrual status, participants were grouped as follows: 1) exercising ovulatory (ExOv, n=20): consistently ovulatory cycles for the duration of the 1-3 menstrual cycle collection period, 2) exercising with inconsistent presentations of subtle menstrual disturbances (ExIncon, n=13): various inconsistent combinations of
ovulatory, luteal phase defects, and anovulatory cycles from cycle to cycle for the duration of the 1-3 menstrual cycle collection period, 3) exercising anovulatory (ExAnov, n=8): consistently anovulatory menstrual cycles for the duration of the 1-3 menstrual cycle collection period, 4) exercising oligomenorrheic (ExOligo, n=20): inconsistent and long menstrual cycle intervals of 36-90 days, and 5) exercising amenorrheic (ExAmen, n=30): no menses for a minimum of 90 days prior to the study and for the duration of the study period.

**Dietary Energy Intake**

Measures of EI were calculated from 3-day diet logs completed during the study period. Participants were provided with a food scale (ECKO Kitchen Scale) and/or food amounts packet. The packet contained diagrams illustrating container sizes, cuts of meat, and various circles and squares which are typically used when estimating portion size for foods. Participants were encouraged to use these scaled diagrams as a guide for describing dimensions and sizes. Also, included in the packet was a sample page of an accurately completed diet record provided as a reference. Participants were asked to record all foods and beverages consumed on 2 weekdays and 1 weekend day. Registered dietitians and/or trained laboratory personnel instructed each subject on how to record EI and then later reviewed diet logs with participants for completeness and accuracy. The nutrient data from the 3-day diet logs were coded and analyzed using Nutritionist Pro (Version 3.1, Axxya Systems, Stafford, TX) or the Nutrition Data System for Research (NDSR 2008 Version; University of Minnesota; Minneapolis, MN).
Exercise Energy Expenditure

Participants completed exercise logs where all purposeful exercise sessions greater than 10 minutes in duration with a heart rate above 90 beats per minute were recorded for a 7 day period. Purposeful exercise included activities such as elliptical, pilates, running, or strength training, but not daily living activities such as house cleaning or walking a dog. Energy expended during these purposeful exercise sessions was measured using the OwnCal feature of the Polar S610 or RS400 heart rate monitors (Polar Electro Oy, Kempele, Finland) (49). The OwnCal feature has been validated for the use in calculating exercise energy expenditure from heart rate (95-96, 107). This feature uses body weight, height, age, gender, VO\textsubscript{2peak}, individual maximum heart rate, individual heart rate in a sitting position, and heart rate during exercise to derive kilocalories from energy expenditure. Actual VO\textsubscript{2peak} values were input into the heart rate monitors to compute exercise energy expenditure. The Polar S601 and RS400 heart rate monitors include rest in their estimation of energy expenditure. To estimate only EEE, we subtracted measured REE (kilocalories/min) from the Polar heart rate monitors estimation of energy expenditure. For purposeful exercise sessions in which participants did not wear the heart rate monitors, the Ainsworth et al. (4, 6) compendiums of physical activities were used to determine the appropriate metabolic equivalent (MET) level for the exercise performed (3). To calculate the energy expended during the exercise session, the MET level was multiplied by the duration (min) of the exercise session. The MET value includes a resting component. To estimate only EEE, we therefore subtracted measured REE (kilocalories/min) from this value. Determination of MET levels from exercise logs from both experimental sites was made by the same research team.
Energy Availability

EA was operationally defined as EI minus EEE relative to kilograms of LBM (EA = (EI – EEE)/LBM) (136) and calculated using the data described above for EI, EEE, and LBM. We calculated EA using the averages of each participant’s values for EI, EEE on workout days only, and the LBM from the DXA scan. EEE represents only those calories attributable to exercise in that the estimate of the calories expended for REE throughout the duration of purposeful exercise sessions was subtracted from the estimate of exercise caloric expenditure using the Polar and or physical activity logs.

Urinary Hormone Measurements

All urine samples were corrected for specific gravity using a hand refractometer (NSG Precision Cells) to account for hydration status (162) which has been reported to perform as well as creatinine correction for adjusting urinary hormone concentrations (162). Microtiter plate competitive enzyme immunoassays were used to measure the daily values for urinary metabolites E1G and PdG as previously reported (60, 64). Urinary LH was measured in samples during the ovulatory phase of the menstrual cycle as determined by visual confirmation of a pre-ovulatory rise in E1G followed by a sustained increase in PdG. LH was determined using a coat-a-count immunoradiometric assay (Siemens Healthcare Diagnostics, Deerfield, IL). The sensitivity of the LH assay was 0.15 mIU/ml and the intra-assay and inter-assay coefficients of variation were 1.6% and 7.1%, respectively.

Serum Measurements

Blood was collected after an overnight fast before 1000 hr once during the study period. Participants were asked to lie supine for at least 15 minutes after which a blood
sample was obtained via venipuncture. Samples were allowed to clot for at least 30 minutes at room temperature and then spun in a centrifuge at 4°C Celsius for 15 minutes at 3000 rpm whereafter serum was transferred into appropriately labeled 1.5 mL microtubules and stored at -80°C Celsius until analysis. Serum samples were assayed in duplicate for total TT₃ using an Immulite chemiluminescent assay (First Generation Immulite 1000, Siemens, Deerfield, IL). The sensitivity of the assay was 35 ng/dL and the intra-assay and inter-assay coefficients of variation were 10.3% and 13.3%, respectively. To determine FAI for screening purposes, total testosterone was measured using a radioimmunoassay kit (Siemens, Los Angeles, CA) through competitive immunoassay. The sensitivity of the assay was 0.14 nmol/L (4.0 ng/dl) and the intra-assay and inter-assay coefficients of variation were 6.4% and 7.5%, respectively. SHBG was assayed in duplicate using a chemiluminescence analyzer (First Generation Immulite 1000, Siemens, Deerfield, IL) through competitive immunoassay. The sensitivity of the assay was 0.2 nmol/L (5.76 ng/dl) and the intra-assay and inter-assay coefficients of variation were 6.4% and 8.7%, respectively.

Statistical Analysis

We calculated sample size based on expected differences and standard deviations from De Souza et al. (62) who demonstrated significant differences in EA (kcal/kg body weight) in exercising women with subtle menstrual cycle disturbances (62). A power coefficient of 0.80 was expected with a sample size of 80 participants with P<0.05 considered to be significant. All variables were tested for outliers and normality using box plot analyses and Kolmogorov-Smirnova tests of normality, respectively. Extreme outliers were not included in our analyses. A 1 way analysis of variance (ANOVA) was
performed to examine differences in EA between menstrual groups. Post hoc testing to reveal where significant differences occurred was performed using t-tests with Fisher's Least Significant Difference test. Chi-square analyses were performed to examine the distribution of menstrual cycle disturbances above and below an EA of 30 kcal/kg LBM. Data are reported as means ± SEM, and $p \leq 0.05$ was considered statistically significant. All data were analyzed using SPSS for Windows (version 18; Chicago, Ill., USA).

Results

Demographic, reproductive, and training characteristics of participants

Demographic, anthropometric, reproductive, and training characteristics for all groups of participants are shown in Table 3-1. The groups were similar with respect to age, height, weight, BMI, body fat (%), fat mass, lean body mass, VO$_{2\text{peak}}$, exercise volume, exercise frequency, and exercise intensity. Post hoc testing showed a later age of menarche in the ExAmen than ExOv ($p=0.004$) and ExIncon ($p=0.007$) groups and in the ExOligo than ExOv ($p=0.015$) and ExIncon ($p=0.019$) groups. Post hoc testing also showed a younger gynecological age in the ExAmen than ExOv ($p<0.001$), ExIncon ($p=0.019$), and ExAnov ($p=0.013$) groups. The ExOligo also showed a younger gynecological age than the ExOv ($p=0.008$) group. The modes of purposeful exercise are represented in Figure 3-1. Running comprised the greatest proportion (21%) of the modes of purposeful exercise when all groups were combined.
Table 3-1. Demographic, anthropometric, reproductive, and training characteristics of participants categorized by exercise and menstrual status.

<table>
<thead>
<tr>
<th></th>
<th>ExOv (n=20)</th>
<th>ExIncon (n=13)</th>
<th>ExAnov (n=8)</th>
<th>ExOligo (n=20)</th>
<th>ExAmen (n=30)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographic</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Characteristics</td>
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<tr>
<td>Age (years)</td>
<td>24.9 ± 1.1</td>
<td>23.6 ± 1.2</td>
<td>24.9 ± 2.2</td>
<td>22.6 ± 0.9</td>
<td>21.6 ± 0.6</td>
<td>0.068</td>
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<tr>
<td>Height (cm)</td>
<td>164.4 ± 1.3</td>
<td>165.5 ± 1.8</td>
<td>166.4 ± 1.0</td>
<td>166.1 ± 1.2</td>
<td>165.9 ± 1.2</td>
<td>0.868</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>57.8 ± 1.3</td>
<td>59.5 ± 1.7</td>
<td>60.2 ± 1.2</td>
<td>58.1 ± 1.4</td>
<td>56.9 ± 1.3</td>
<td>0.594</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.4 ± 0.4</td>
<td>22.1 ± 0.3</td>
<td>21.8 ± 0.5</td>
<td>21.0 ± 0.4</td>
<td>20.7 ± 0.4</td>
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<tr>
<td>Body fat (%)</td>
<td>25.2 ± 0.8</td>
<td>26.4 ± 1.3</td>
<td>26.0 ± 1.7</td>
<td>25.0 ± 1.2</td>
<td>25.0 ± 1.0</td>
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<td>Fat mass (kg)</td>
<td>14.5 ± 0.6</td>
<td>15.7 ± 1.0</td>
<td>15.5 ± 1.3</td>
<td>14.2 ± 0.8</td>
<td>14.4 ± 0.7</td>
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<td>Lean body mass (kg)</td>
<td>41.5 ± 0.9</td>
<td>41.2 ± 0.6</td>
<td>43.5 ± 0.7</td>
<td>42.0 ± 1.2</td>
<td>40.2 ± 0.8</td>
<td>0.318</td>
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<tr>
<td><strong>Reproductive</strong></td>
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<td>Characteristics</td>
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</tr>
<tr>
<td>Age of menarche (years)</td>
<td>12.2 ± 0.3</td>
<td>12.1 ± 0.4</td>
<td>12.4 ± 0.5</td>
<td>13.4 ± 0.4&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.5 ± 0.3&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.006</td>
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<td>Gynecological age (years)</td>
<td>12.7 ± 1.1</td>
<td>11.5 ± 1.2</td>
<td>12.4 ± 2.3</td>
<td>8.8 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.0 ± 0.6&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;b&lt;/sup&gt;,&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.002</td>
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<tr>
<td>Characteristics</td>
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<td></td>
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<tr>
<td>VO₂&lt;sub&gt;peak&lt;/sub&gt; (ml/kg/min)</td>
<td>47.3 ± 1.1</td>
<td>44.5 ± 1.4</td>
<td>46.3 ± 2.4</td>
<td>45.4 ± 0.9</td>
<td>45.4 ± 1.6</td>
<td>0.726</td>
</tr>
<tr>
<td>Exercise volume (min/week)</td>
<td>337.6 ± 46.2</td>
<td>360.2 ± 56.8</td>
<td>337.4 ± 63.3</td>
<td>376.6 ± 60.7</td>
<td>357.2 ± 50.8</td>
<td>0.990</td>
</tr>
<tr>
<td>Exercise frequency (sessions/week)</td>
<td>4.2 ± 0.4</td>
<td>4.7 ± 0.5</td>
<td>5.1 ± 0.7</td>
<td>4.6 ± 0.4</td>
<td>4.4 ± 0.3</td>
<td>0.774</td>
</tr>
<tr>
<td>Exercise intensity (kcal/min)</td>
<td>6.1 ± 0.5</td>
<td>4.9 ± 0.4</td>
<td>4.9 ± 1.0</td>
<td>5.2 ± 0.5</td>
<td>6.2 ± 0.4</td>
<td>0.162</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM.

ExOv, exercising ovulatory; ExIncon, exercising inconsistent menstrual cycles; ExAnov, exercising anovulatory; ExOligo, exercising oligomenorrhea; ExAmen, exercising amenorrhea

<sup>a</sup>, p< vs. ExOv; <sup>b</sup>, p< vs. ExIncon; <sup>c</sup>, p< vs. ExAnov; <sup>d</sup>, p< vs. ExOligo
Figure 3-1. Pie chart representing the modes of purposeful exercise among all the exercising participants with ovulatory, inconsistent, anovulatory, oligomenorrheic, and amenorrheic menstrual cycles during the study period.
Menstrual Status

Composite graphs depicting the urinary metabolites, E1G and PdG, of the ExOv, ExIncon, ExAnov, ExOligo, and ExAmen menstrual cycles are presented in Figure 3-2. In the ExOv (Figure 3-2A) group, a mid-cycle E1G peak (>35 ng/ml) followed by a rise in PdG (>5 µg/ml) during the luteal phase was observed. In the ExIncon group (Figure 3-2B), a combination of a minimal rise in E1G (<35 ng/ml), inadequate PdG concentrations (<5 µg/ml), and shortened luteal phases (<10 days) was observed. In the ExAnov group (Figure 3-2C), a minimal rise in E1G followed by inadequate PdG (<2.5 µg/ml) concentrations was observed. In the ExOligo group (Figure 3-2D), elevated and erratic E1G concentrations were observed. Lastly, in the ExAmen group (Figure 3-2E), E1G and PdG concentrations were chronically suppressed. The average duration of amenorrhea for the ExAmen group was 252.2 ± 35.1 days.
Day of Cycle
1 3 5 7 9 11 13 15 17 19 21 23 25 27

E1G (ng/ml)
0 25 50 75 100 125

PdG (ug/ml)
0 2 4 6 8 10

Day of Cycle
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33

E1G (ng/ml)
0 25 50 75 100 125

PdG (ug/ml)
0 2 4 6 8 10

D
Figure 3-2. Composite graphs representing the menstrual characteristics of exercising ovulatory (A), exercising with inconsistent menstrual cycles (B), exercising anovulatory (C), exercising oligomenorrheic (D), and exercising amenorrheic (E) participants. The estrone-1-glucuronide (E1G) (ng/ml) and pregnanediol-3-glucuronide (PdG) (µg/ml) data for the exercising ovulatory and exercising with inconsistent menstrual cycle participants are aligned by the day of the LH peak, defined as day 0. The number of days depicted for the exercising ovulatory, exercising with inconsistent menstrual cycles, exercising anovulatory, and exercising oligomenorrheic participants represents the mean cycle length for these participants. The number of days depicted for the exercising amenorrheic participants represents the menstrual collection period, 28 days. Data are reported as mean ± SEM of the one to three menstrual cycles per participant such that each participant’s data are represented once in the figure.

Energy Availability

The EA of individual participants is presented in Figure 3-3. Low EA (<30kcal/kg LBM) was observed in 5 of 20 (25%) in the ExOv group, 4 of 13 (31%) in the ExIncon group, 0 of 8 (0%) in the ExAnov group, 5 of 20 (25%) in the ExOligo group, and 15 of 30 (50%) in the Ex Amen group. An EA of 30 kcal/kg LBM did not discriminate menstrual status in that the proportion of exercising women who had ovulatory menstrual
cycles did not differ from the proportion of women with EAMD when grouped according to whether they had an EA above or below 30 kcal/kg LBM ($\chi^2=0.557$, p=0.456). EA and its components are presented in Figure 3-4. There was no difference in mean EA among the menstrual groups ((F=1.247, p=0.297) (Figure 3-4A)). No differences in mean EI (Figure 3-4B), EEE (Figure 3-4C), or LBM (Figure 3-4D) were observed between the groups.
Figure 3-3. Dot histogram representing the individual energy availability of the exercising with ovulatory menstrual cycles (ExOv), exercising with inconsistent menstrual cycles (ExIncon), exercising with oligomenorrhea (ExOligo), and exercising with amenorrhea (ExAmen) participants during the study period. Black bar denotes group mean.
Figure 3-4. Bar graphs representing the energy availability (A), dietary energy intake (B), exercise energy expenditure (C), and lean body mass (D) of the exercising with ovulatory menstrual cycles (ExOv), exercising with inconsistent menstrual cycles (ExIncon), exercising with oligomenorrhea (ExOligo), and exercising with amenorrhea (ExAmen) participants during the study period. Data are reported as mean ± SEM.
Metabolic Status

Data for indices of energy status are presented in Figure 3-5. ANOVA revealed a difference in REE (kg/LBM) among groups (F=3.103, p=0.020). Post hoc testing showed a lower REE (kg/LBM) in the ExAnov (p=0.022), ExOligo (p=0.002), and ExAmen (p=0.007) than the ExOv group (Figure 3-5A). ANOVA revealed a difference in ratio of REE/pREE among groups (F=4.046, p=0.005). Post hoc testing showed a lower ratio of REE/pREE in the ExOligo (p=0.004) and ExAmen (p<0.001) than ExOv group (Figure 3-5B). (Figure 3-5C). ANOVA revealed a difference in TT₃ concentrations among exercising groups (F=2.508, p=0.048). Post hoc testing showed lower TT₃ concentrations in the ExAmen than ExOv (p=0.040) and ExAnov groups (p=0.009).
Figure 3-5. Bar graphs representing resting energy expenditure (kcal/kg LBM) (A), resting energy expenditure ratio (B), and total triiodothyronine (ng/dL) (C) of the exercising with ovulatory menstrual cycles (ExOv), exercising with inconsistent menstrual cycles (ExIncon), exercising with oligomenorrhea (ExOligo), and exercising with amenorrhea (ExAmen) participants during the study period. \(^a\), \(p<0.05\) vs. ExOv. \(^b\), \(p<0.05\) vs. ExAnov. Data are reported as mean ± SEM.
Discussion

An important feature of this study is that the data analyzed likely represent perhaps the largest dataset available in exercising women to date where menstrual disturbances have been confirmed with measurements of estrogen, progesterone, and luteinizing hormone metabolites from daily urine samples. The current study is the first to test the concept that EA threshold of 30 kcal/kg LBM, defined similarly as Loucks et al. (144), discriminates ovulatory menstrual cycles from exercise associated menstrual disturbances (EAMD), particularly amenorrhea, when assessed with diet logs, exercise logs, and heart rate monitoring in a large population of trained, exercising women studied under free living conditions. This study was also the first to examine whether, below an EA of 30 kcal/kg LBM, levels of EA become lower as menstrual cycle disturbances progress in severity from inconsistent combinations of ovulatory, luteal phase defects, and anovulatory cycles, to anovulatory menstrual cycles, oligomenorrhea, and finally to amenorrhea.

Our results add to the small but growing number of findings from studies attempting to determine the association between EA and ovarian function in exercising women in a field setting (196, 204). An important contribution of the current study is that EA, defined as EI minus EEE relative to kilograms of LBM (144), did not discriminate menstrual status in a large population of trained, exercising women with varying menstrual cycles and did not discriminate amenorrhea from menstrual cycles with bleeding intervals. This finding is similar to other much smaller studies which have examined EA (204) or its components (121, 170, 261) in premenopausal exercising women. Schaal et al. (204) did not observe differences in EA between eumenorrheic and
amenorrheic endurance trained athletes (29 vs. 18, p=0.21). Moreover, in studies which examined the components of EA (121, 170, 261), neither Wilmore et al. (261), Laughlin and Yen (121), nor Myerson et al. (170) observed significant differences in EI, EEE, or body composition between amenorrheic and eumenorrheic athletes. In a previous report (196), we showed lower mean EA in exercising women with amenorrhea than in women with ovulatory menstrual cycles. We attribute this observed difference in EA in association with menstrual status to a smaller sample size (n=25) as we did not observe a similar difference in EA when a larger population of exercising women with varying menstrual status were included in the analyses (n=91).

We observed lower mean EA values in comparison to those reported in the literature for sedentary women. These lower values may be attributable to an uncoupling mechanism between EI and EEE. Findings from studies examining the coupling between EI and EEE (101, 108, 171, 230, 253-254) suggest that inadvertent under eating leads to low EI. For example, only a 30% compensation of exercise induced energy expenditure, with the degree of compensation varying considerably, was observed in response to increase levels of physical activity in young, lean exercising females and males. The lack of an increase in EI in response to an acute exercise-induced energy expenditure i.e., inadequate compensation has been observed in young, obese, and non-obese men and women (108, 230, 253-254). The above studies (108, 171, 230, 253-254) suggest that inadvertent dietary compensation to match EEE may have contributed to the lower overall mean EA (35 kcal/kg LBM) observed in our exercising women when compared to the EA of sedentary women deemed to be in energy balance (144) than EA values we calculated from the reported EI, EEE, and body composition of the sedentary women of
In our study, we examined the relationship between EA and EEE in an attempt to understand the coupling between EI and EEE and lower EA values. We observed a significant negative relationship between EA and EEE which may suggest that inadequate dietary compensation increases as the volume of exercise increases.

Although our exercising women exhibited a lower overall mean EA than those of sedentary women (121, 170), our EA values (31-40 kcal/kg LBM) are higher than other studies. For example, using EI, training mileage, and fat free mass from several studies of female amenorrheic runners, Loucks et al. (134) estimated EA values ranging from 12 to 29 kcal/kg FFM in female athletes. Schaal et al. (204) and Reed et al. (196) observed EA values below 30 kcal/kg LBM in exercising women with amenorrhea, albeit in a much smaller sample of athletes than our investigation. Lastly, using reported EI, EEE, and FFM, we estimated an EA value of 28 kcal/kg FFM for the amenorrheic runners of Myerson et al. (170). Since we observed a negative relationship between EA and EEE, suggestive of the presence of an uncoupling mechanism between EI and EEE (108, 230, 253-254), it is possible that inadequate EI compensation to match lower EEE resulted in higher EA values (>30 kcal/kg LBM) when compared to other studies where the training volume of the participants and thus EEE was higher (204) than in our exercising women. Using this rationale, the higher mean EEE of Schaal et al. (204) (1300 kcal/d) and Myerson et al. (170) (537 kcal/d) would explain the lower EA these investigators reported (170, 204). These investigators (170, 204) also observed negative eating attitudes including high body shape concerns and total eating disorder scores in their female athletes. Aberrant eating attitudes have been shown to predispose women to
consume less energy (57, 84, 196). It is therefore also possible that higher EEE in combination with negative eating attitudes might have contributed to the lower EA values documented in these amenorrheic exercising women (170, 204).

It is possible that changes in the components of energy balance not captured in the EA calculation contributed to the inability of EA to discriminate menstrual status. The components of 24 hour energy expenditure (TDEE) include REE, thermic effect of food, non-exercise activity thermogenesis (NEAT), and EEE (128). The calculation of EA, defined as EI minus EEE relative to kilograms of LBM, does not account for REE, themic effect of food, or NEAT which can represent approximately 80% of TDEE. Increased TEF of 24 % has been observed in anorexic patients with amenorrhea (165). Moreover, increases and decreases in NEAT have been shown in a state of positive and negative EB, respectively (125, 129, 199). Changes in these parameters of EB are not accounted for in the calculation of EA and therefore might explain the lack of observed difference in EA between our exercising women with varying menstrual status. That is, increased NEAT and TEF may have produced an energy deficit resulting in the lower REE (kcal/kg LBM) we observed in our women with severe menstrual disturbances. Previous studies have similarly reported lower REE (kcal/kg LBM) in energy deficient exercising women with amenorrhea (58, 124, 168, 170). Alternatively, factors such as psychological stress (18, 184, 255) or hyperandrogenemia (88, 198) might account for the observed EAMD suggesting that EA may not play a role in the menstrual disturbances observed in some proportion of our exercising women.

In addition to lower REE (kcal/kg LBM), we observed lower TT₃ concentrations and ratio of REE/pREE in exercising women with severe menstrual disturbances. These
metabolic adaptations suggest that our exercising women with amenorrhea were energy deficient as suppression of TT$_3$, REE, and menstrual function were apparent in these women (58, 168, 170, 247). Previous cross sectional studies have similarly documented lower TT$_3$ concentrations (58, 121, 142), REE (58, 124, 168), and ratio of REE/pREE (66) in exercising women with severe menstrual disturbances. These metabolic adaptations further suggest that the methods used in the current study to assess EA did not capture differences in energy status between our women with ovulatory menstrual cycles and those with severe menstrual disturbances. Because these more objective laboratory methods suggest adaptations to conserve energy in association with menstrual disturbances in our subjects, it is therefore more likely that the our combination of laboratory and field methods used to assess the EI, EEE, and LBM may have contributed to a higher degree of variability in the estimate of EA and thus the lack of observed differences in EA. This increased variability and reliance on self reported food intake and exercise behavior and its impact on the measurement of EA is likely more relevant to our findings than the physiological link between EA and reproductive function since several studies manipulating EA (144), EI and EEE (32, 193, 256-257, 259-260) or EI alone (137, 178, 188-190, 210) support the causal role EA in the neuroendocrine regulation of reproductive function.

In short term studies manipulating EI and EEE which lead to significant disruptions in LH pulsatility below an EA of 30 kcal/kg LBM, Loucks et al. (137, 144) used a clinical dietary product (Ensure, Ross Laboratories, Columbus, OH) to control EI and indirect calorimetry to assess EEE. Conversely in the current study, we assessed EI and EEE using more conventional methods for exercising women. Namely, self-reported
diet logs, exercise logs, and heart rate monitoring were used to assess EA. Several studies have shown inaccuracies when measuring EI with self-reported diet logs, particularly related to under reporting (93, 112, 203) and EEE with self-reported exercise logs (47, 112). Heart rate monitoring has been shown to estimate total EE within -4.0 to 11.4% in females when compared to doubly labeled water which is the gold standard for measuring EE in free-living conditions. Although heart rate monitoring provides an objective assessment of EEE, participants must be compliant and wear the monitors for the duration of all their exercise training sessions. It is therefore possible that the inaccuracies associated with self-reported diet logs, exercise logs, and heart rate monitoring in assessing EI and EEE may have contributed to the lack of observed differences in EA among our exercising women with varying menstrual status.

Conclusion

This study was the first to show that EA, defined as EI minus EEE relative to kilogram of LBM, did not discriminate menstrual status in a large population of exercising women when using conventional methods to assess EA including self-reported diet logs, exercise logs, and heart rate monitoring. In addition, we showed that EA levels do not significantly decline as menstrual disturbances increase in severity. Lower TT3 concentrations, REE, and ratio of REE/pREE were observed in our exercising women with menstrual disturbances indicating that our participants likely were exhibiting adaptations to chronic energy deficiency. We therefore propose that the above methods used to assess EA largely contributed to the lack of observed differences in EA in our
large population of exercising women. Future studies should focus on identifying ways to improve field measures of EI and EEE and whether more precise techniques to measure these components of improve the ability of EA as a variable in discriminating menstrual status and consequently those at risk for the Female Athlete Triad.
Chapter 5
The role of energy availability in the reversal of menstrual disturbances in exercising women

Introduction

Normal reproductive function in a variety of mammalian species is dependent upon the pulsatile release of gonadotropin-releasing hormone (GnRH) from the arcuate nucleus of the hypothalamus (191). The neural mechanisms controlling the release of gonadotropin-releasing hormone response to short and long term changes in energy availability (EA) (247). Findings from several studies manipulating dietary energy intake (EI) and exercise energy expenditure (EEE) (32, 193, 256-257, 259-260) or dietary energy intake alone (137, 178, 188-190, 210) support the causal role of low EA in the development of reproductive disturbances in exercising females. Regarding the reversal of reproductive disturbances when EA is restored, studies in animals (258) and humans (83, 86, 163, 258) have demonstrated the effectiveness of either increased dietary energy intake (83, 86, 163, 258), decreased exercise energy expenditure (249), or the manipulation of both (73, 115) to improve indices of reproductive function. It is important to note that while changes in body weight are generally concomitant with alterations in reproductive function resulting from changes in dietary energy intake and or energy expenditure, body weight is not always an accurate reflection of changes in energy balance (257, 259) and thus quantifying changes in the components of energy balance is important.
While the physiological importance of EA in both the induction and reversal of reproductive dysfunction has been demonstrated, an important practical consideration that has not been adequately explored is the magnitude of change in EA necessary to either induce or reverse menstrual disturbances in exercising women. Understanding whether a particular threshold of EA exists below which reproductive disturbances ensue, or above which reproductive recovery is stimulated, is also a key translational question that must be addressed. If such studies are performed, correctional nutrition strategies could be developed for special populations of women susceptible to energetically-related reproductive dysfunction such as female military recruits (120, 207-208), exercising women (57-58, 64, 68, 98, 196), and/or women with disordered eating behaviors (42, 57, 84, 241). Since quantifying EA involves the estimation of both energy intake and expenditure sides of the energy balance equation, studies must include careful measurement of these components.

To this end, EA has been operationally defined by Loucks et al. (144) as EI minus EEE relative to kilograms of lean body mass (LBM) i.e., \( EA = EI - EEE / \text{kg LBM} \). Reductions in LH pulsatility (144) and unfavorable changes in markers of bone turnover (103) have been observed below an EA threshold of 30 kcal/kg LBM in previously sedentary women during short-term manipulations of EI and EEE in a controlled laboratory setting. To date, no studies have tested the effectiveness of this threshold in the development of menstrual disturbances caused by changes in diet and exercise, nor have any studies been performed to test the effectiveness of this threshold as a key level of EA associated with the reversal of menstrual disturbances in exercise and or diet related amenorrhea or oligomenorrhea. Such studies are necessary to develop effective,
non-pharmacological treatment strategies to either prevent or reverse reproductive
dysfunction in the aforementioned groups of women.

We conducted a randomized controlled trial to test the effectiveness of increasing
food intake without altering training habits to reverse amenorrhea or oligomenorrhea over
a 12 month time period. This study represents a sub-study of this randomized controlled
trial that specifically addresses whether the particular expression of EA, defined at EI
minus EEE relative to kilograms of LBM, is a useful predictor of the reversal of exercise
associated menstrual disturbances in exercising women. The purpose of the current study
was to test the following hypotheses: 1) women with exercise associated menstrual
disturbances (EAMD) who display reproductive recovery will be characterized by a
greater increase in EA (kcal/kg LBM) when compared to women with EAMD who do not
display reproductive recovery, and 2) a greater proportion of women with EAMD who
display reproductive recovery will exhibit an EA (≥ 30 kcal/ kg LBM) when compared to
trained premenopausal women with EAMD who do not display reproductive recovery.

Methods

Study Design

The current study represents a sub-study of a randomized controlled trial designed
to test whether a 12-month intervention of increased caloric intake would improve indices
of bone health and menstrual status in exercising women who suffer from severe EAMD,
including oligomenorrhea (long and inconsistent menstrual cycles of 36-90 days) and
functional hypothalamic amenorrhea (the absence of menses for >90 days). The study
was conducted at two sites, first at the University of Toronto and then at the Pennsylvania
State University over 6 years. At each site exercising women with normal menstrual cycles and exercising women with menstrual disturbances were recruited. The women with normal menstrual cycles were studied to serve as a reference control group; data on these participants are not included in the current study. The study design is illustrated in Figure 4-1. The current study includes data from all women with severe EAMD who completed 6 months of an intervention designed to increase caloric intake 20-30% above baseline energy requirements. Participants were randomly assigned to a treatment group (EAMD + calories; n=11) or a control group (EAMD controls; n=10) at the beginning of the intervention after a baseline monitoring period. For the purposes of the current study, the exercising women with oligomenorrhea and amenorrhea were retrospectively re-categorized into one of two groups regardless of their initial group assignment: 1) those who displayed reproductive recovery between months one and six of the intervention, and 2) those who did not display reproductive recovery between months one and six of the intervention. Two categories of reproductive recovery were defined, the resumption of menses and a second category of recovery that represents a more “robust” functional recovery, i.e., the resumption of menses preceded by ovulation. For each of these categories our participants were divided into those who met the criteria for recovery and those who showed no evidence of reproductive recovery. For a detailed description of the indices used to categorize reproductive recovery, please see Classification of Intervention Menstrual Status below.

Repeated measures of dietary energy intake (kcal/d), body weight (kg), body composition (kg LBM), exercise energy expenditure (kcal/d), training volume (exercise minutes), and daily urinary metabolites (estrogen, progesterone and LH) were collected
during the study. To corroborate changes in metabolic status using an objective laboratory measure, serum total triiodothyronine (TT$_3$) was repeatedly assessed. The primary outcome measures pertaining to the current sub-study include detailed assessment of menstrual status by daily urinary hormones and measures of EA (kcal/kg LBM) and TT$_3$ (ng/mL). Secondary outcome measures include body weight, fat mass, lean mass and TT$_3$. 
Figure 4-1. Study procedures for screening through intervention week 21. CBC, Complete Blood Count; LH, luteinizing hormone, FSH, follicle stimulating hormone, DHEAS, dehydroepiandrosterone sulfate; E1G, estrone-1-glucuronide; PdG, pregnanediol-glucuronide
Participants

Participants were recruited by newspaper advertisements, fliers, and classroom announcements targeting physically active women for a study examining the impact of increased caloric intake on bone health and menstrual cyclicity. Recruitment advertisements targeted exercising women with normal and abnormal menstrual cycles. Inclusion criteria for this study were: 1) age 18-35 years, 2) body mass index (BMI) 16-25 kg/m$^2$, 3) weight stable (± 2kg) for the past 6 months, 4) no history of any serious medical conditions, 5) no current clinical diagnosis of an eating or psychiatric disorder, 6) non-smoking, 7) no medication use that would alter metabolic or reproductive hormone concentrations, 8) ≥ 3 hrs/wk aerobic exercise, 9) no history of a clinical diagnosis of polycystic ovarian syndrome (PCOS), or a free androgen index (FAI), calculated as (total testosterone (nmol/L) / sex hormone binding globulin (SHBG) (nmol/L))*100) (200) > 6, since an FAI greater than 6.0 has been reported to be consistent with hyperandrogenemia (88, 198). In addition, an FAI greater than 6.0 represents values greater than three standard deviations from the mean of 1.6 in our reference population which consisted of healthy premenopausal exercising women with documented ovulatory menstrual status (61, 64) by the assessment of daily urinary hormone measurement (61, 64).

Screening Procedures

During an initial visit, participants were informed of the purpose, procedures, and potential risks of participation in the study before signing an informed consent approved by either the Human Ethics Board at the University of Toronto or Biomedical Institutional Review Board at the Pennsylvania State University. Once consent was
obtained, height and weight were measured, and participants completed questionnaires to assess demographics, medical history, exercise history, menstrual history, eating behaviors (82, 222), bone health, and psychological health (29, 43, 69, 228). A physical exam was performed by a clinician to determine overall health and check for physical signs and symptoms of disordered eating and PCOS such as acne or hirsutism. A fasting blood sample was analyzed for a complete blood count, a basic chemistry panel, and an endocrine panel which included measures of LH, follicle stimulating hormone, thyroid stimulating hormone, thyroxine, prolactin, dihydroepiandrosterone (Quest Diagnostics, Pittsburgh, PA), total testosterone, and SHBG to rule out illness or endocrine and metabolic disease. A clinical psychologist or licensed clinical social worker interviewed each subject to exclude those women experiencing major psychiatric disorders including depression or clinical eating disorders. Participants met with a registered dietitian after completing a 3-day diet log (2 weekdays and 1 weekend day) to discuss eating patterns and food preferences and to assess likelihood for compliance to the study protocol with respect to increased food intake. Dual-energy x-ray absorptiometry (DXA) scans of the total body, lumbar spine, and dual femur were performed to assess bone mineral density (BMD) and body composition.

Figure 4-2 depicts the progression of subjects throughout the study. One hundred and sixty-four subjects were assessed for eligibility for the study during baseline and screening procedures. Sixty-seven women were eliminated during the screening period and baseline period, i.e. 12 for medical reasons, 6 for BMI outside inclusion criteria, 2 for age outside inclusion range, 2 for not meeting exercise requirements, 2 decided to go on oral contraceptives, 1 was not weight stable, 8 for time commitment reasons, and 34 for
other reasons. Thirty-nine women were assigned to the ovulatory menstrual function group and were not assessed in the current study. Thirty women were assigned to the EAMD control group, and 28 to the EAMD + calories group. Thirty-three women were eliminated during the intervention for many reasons, including 7 for time commitment reasons, 4 moved away, 2 were unwilling to increase caloric intake, 3 decided to go on oral contraceptives, 1 was physician recommended, 2 discovered medical conditions, 2 had high FAI, and 16 for other reasons. Twenty-one women completed 6 months of the study. However, one subject with oligomenorrhea was not included in the recovery of reproductive function (described below), therefore the analysis for the recovery of reproductive function is based on a sample size of twenty women.
Figure 4-2. Consort diagram showing flow of participants through the study.
Classification of Baseline Menstrual Status

The initial classification of menstrual status was based on self-reported menstrual histories, the results of a physical examination, urinary estrone-1-glucuronide (E1G), pregnanediol-glucuronide (PdG), and LH profiles, and other endocrine measures described below. Menstrual calendars were used to chart menstrual symptoms such as cramps, bleeding, spotting, discharge, etc. Participants collected first morning urine samples throughout a 4 week baseline monitoring period. Oligomenorrhea was confirmed if menses occurred at intervals of 36-90 days and if participants self-reported 6 or less menstrual cycles in the last year prior to the intervention. Functional hypothalamic amenorrhea was assessed by confirming a negative pregnancy test, no menses in the past 90 days, and documentation of suppressed E1G and PdG profiles during the baseline monitoring period.

Intervention Procedures

Participants randomly assigned to the treatment group (EAMD + calories) were counseled to increase their caloric intake 20-30% above baseline energy requirements while maintaining their usual exercise training regime. Participants in the EAMD + calories group could choose to increase their caloric intake through the use of foods they preferred and or nutritional/sports energy supplements that contained approximately 300 calories which were provided by the research staff. EAMD controls were asked to keep their caloric intake and exercise training the same as it is when they signed up for the study. A registered dietician met with the participants from both groups every other week for the first three months and then once a month for remaining months of the intervention to review the participants’ diet and provide strategies to meet their target
calorie intake. Participants also met with a clinical psychologist or licensed clinical social worker every other week for the first three months and then once a month for the remaining months of the intervention to monitor participants’ general psychological health and provide assistance in helping to implement lifestyle changes that participants decided to make in the context of the intervention. Repeated measures of dietary intake (kcal/d), body weight (kg), body composition (kg LBM), training energy expenditure (kcal/d), training volume (exercise minutes), serum hormones (total triiodothyronine (TT₃)), and daily urinary metabolites (E1G, PdG, and mid-cycle LH) were collected during the intervention (Figure 4-1).

**Classification of Intervention Menstrual Status**

To describe reproductive recovery, we grouped participants based on two definitions of recovery that ranged in physiological and clinical relevance. For each of the following two categories describing reproductive recovery, each participant was deemed to either have met the recovery of menstrual function criteria during the intervention or have exhibited no evidence of reproductive recovery. The definitions used for each category differed somewhat between women deemed to exhibit functional hypothalamic amenorrhea vs. oligomenorrhea. The first category (Category 1) was entitled “recovery of menses.” The successful recovery of menses in women with exercise associated functional hypothalamic amenorrhea at baseline was defined as the first occurrence of menstrual bleeding during the intervention. Thus, a participant received a “yes” for this category if she experienced menses in the first six months or a “no” if she did not. The successful recovery of menses in women with exercise associated oligomenorrhea at baseline was defined as an increase in the frequency of menses during the first 6 months
of the intervention compared to the frequency of menses as determined by self-report during the 6 months prior to the intervention. Thus, a participant received a “yes” for this category if she experienced an increased frequency of menses in the first six months or a “no” if she did not. One subject with oligomenorrhea was not included in the Category 1 analysis because, although she had a 45 day baseline menstrual cycle and subsequently demonstrated six occurrences of menses during the intervention, she reported 6 menstrual cycles in the last 6 months during her menstrual history.

The second category (Category 2) was entitled “recovery of menses preceded by ovulation.” Participants were scored a “yes” for this category if the resumption of menses was preceded by ovulation based on increases in urinary estrone-1-glucuronide (E1G, above 35 ng/ml), pregnanediol-3-glucuronide (PdG, above 2.5 µg/ml), and mid-cycle LH (above 25 mIU/ml) concentrations (61, 64) or a “no” if participants exhibited no evidence of reproductive recovery. Six women were not included in this category tabulation because two ovulated during their baseline cycle (i.e. oligomenorrheic participants) and thus evidence of ovulation during the intervention could not be deemed to be an improvement in menstrual status for these participants and 4 additional participants that displayed evidence of reproductive recovery i.e. recovered menses that was not preceded by ovulation based on the above criteria.

**Anthropometrics**

Total body weight was measured by a digital scale in the laboratory to the nearest 0.01 kg wearing t-shirt and gym shorts during screening, each week of the 4 week baseline period, and intervention weeks 1, 5, 9, and 21. BMI was calculated as a ratio of weight to height (kg/m²) during screening, each week of baseline, and intervention weeks
Baseline values for body weight and BMI were the average of all screening and baseline measurements. Height was measured to the nearest 1.0 cm without shoes during the screening period. Body composition, including percent body fat, fat mass (FM), fat free mass (FFM), and LBM was analyzed during week 3 of baseline and intervention weeks 1, 5, 9, and 21 by a certified technician using DXA. The majority of participants were scanned on either a GE Lunar Prodigy DXA scanner (n=11) (GE Lunar Corporation, Madison, WI, enCORE 2002 software, version 6.50.069) or a GE Lunar iDXA scanner (n=19) (General Electric Lunar Corporation, Madison, WI, enCORE 2008 software version 12.10.113). Remaining participants were scanned on a Hologic QDR4500 DXA scanner (n=2) (Hologic Inc., Bedford, MA). Consistent with the International Society of Clinical Densitometry guidelines, cross calibration studies were performed to remove systematic bias between the systems. For the cross calibration study between the Lunar Prodigy and Lunar iDXA, fourteen participants were scanned in triplicate on both machines. The majority (n=8) were scanned on both machines within 5 days; however, there was approximately one month between scans for some participants (n=6). The values for body composition obtained on each scanner were found to be highly correlated with no significant difference between the population mean values. For the cross calibration study between the Hologic QDR4500W and the Lunar iDXA, thirty-two participants were scanned in duplicate on both machines on the same day. Equations were derived using simple linear regression to remove biases, and body composition values obtained from both the Lunar Prodigy and the Hologic QDR-4500W were calibrated to the Lunar iDXA.
Exercise Testing

Peak aerobic capacity (VO$_{2peak}$) was measured on a treadmill by indirect calorimetry using an on-line MedGraphics Modular VO$_2$ System (St Paul, MN) or SensorMedics Vmax metabolic cart (Yorba Linda, Calif., USA) during week 3 of the baseline menstrual cycle and intervention week 21 using indirect calorimetry and the modified Åstrand protocol (9, 58, 196).

Resting Energy Expenditure

Resting energy expenditure (REE) was measured by indirect calorimetry using a Sensormedics Vmax metabolic cart (Yorba Linda, CA) during week 3 of baseline and intervention weeks 1, 5, 9, and 21. Participants reported to the lab at approximately 0800 h having fasted for 12 hours and refrained from exercise, alcohol, and caffeine for 24 hours. After a 45 minute rest period, a ventilated hood was placed on the participants, and REE was measured for 30-45 minutes. Oxygen consumption (VO$_2$; mL/min) and carbon dioxide production (VCO$_2$; mL/min) were measured every 30 seconds. To calculate REE, data for VO$_2$ and VCO$_2$ were only used if steady state was attained. Steady state was achieved when the volume of expired air and VO$_2$ were not varying by more than 10% and when the respiratory quotient was not varying by more than 5%. We then only averaged those consecutive data points that met these criteria. REE was calculated using the Weir equation (252) REE (kcal/d) = [3.94(VO$_2$) +1.11(VCO$_2$)] x1.44. In our laboratory, the coefficient of variation for REE measurements, established in 81 participants age 18-35 yrs who underwent two repeated measurements about two weeks apart is 5.3±0.7%. This is within reported literature values for healthy adults aged 19-51 years (44).
Dietary Energy Intake

Dietary energy intake was assessed from 3-day diet logs completed during week 3 of baseline and intervention weeks 1, 5, 9, 13, 17, and 21. Participants were provided with a food scale and food amounts packet. The packet contained diagrams illustrating container sizes, cuts of meat, and various circles and squares which are typically used when estimating portion size for foods like bowls of cereal. Participants were encouraged to use these scaled diagrams as a guide for describing dimensions and sizes. Also, included in the packet was a sample page of an accurately completed diet log provided as a reference. Participants were asked to record all foods and beverages consumed on 2 weekdays and 1 weekend day, including the time and location of every eating episode. Registered dietitians trained each subject how to record dietary intake accurately. The nutrient data from the 3-day diet logs were coded and analyzed using the Nutrition Data System for Research (NDSR 2008 Version; University of Minnesota; Minneapolis, MN).

Exercise Energy Expenditure

Participants completed exercise logs where all purposeful exercise sessions greater than 10 minutes in duration with a heart rate above 90 beats per minute were recorded for a 7 day period. Purposeful exercise included activities such as use of the elliptical machine, running, cycling, or strength training, but not daily living activities such as house cleaning or walking a dog. Energy expended during these purposeful exercise sessions was measured using the OwnCal feature of the Polar S610 or RS400 heart rate monitors (Polar Electro Oy, Kempele, Finland) (49). The OwnCal feature has been validated for the use in calculating exercise energy expenditure from heart rate (95-96, 107). This feature uses body weight, height, age, gender, VO$_2$ peak, individual
maximum heart rate, individual heart rate in a sitting position, and heart rate during exercise to derive kilocalories from energy expenditure. Actual $\text{VO}_2\text{peak}$ values were input into the heart rate monitors to compute exercise energy expenditure. The Polar S601 and RS400 heart rate monitors include rest in their estimation of energy expenditure. To estimate only EEE, we subtracted the most recently measured REE (kilocalories/min) from the Polar heart rate monitors estimation of energy expenditure. For purposeful exercise sessions in which participants did not wear the Polar S610 or RS400 heart rate monitors, the Ainsworth et al. (4, 6) compendiums of physical activities were used to determine the appropriate metabolic equivalent (MET) level for the exercise performed (3). To calculate the energy expended during the exercise session, the MET level was multiplied by the duration (min) of the exercise session. The MET value includes a resting component. To estimate only EEE, we subtracted the most recently measured REE (kilocalories/min) from this value. Determination of MET levels from exercise logs from both experimental sites was made by the same individual. The heart rate monitors were updated as new values for $\text{VO}_2\text{peak}$ and body weight were obtained.

**Energy Availability**

EA was operationally defined as dietary energy intake minus exercise energy expenditure relative to kilograms of lean body mass ($\text{EA} = (\text{EI} - \text{EEE})/\text{LBM}$) (136) and calculated using the data described above for dietary energy intake, exercise energy expenditure, and anthropometrics. EA was measured during week 3 of baseline and intervention weeks 1, 5, 9, 13, 17, and 21 using the most recently collected data for EI, EEE, LBM, and REE. To calculate the change in EA during the 6 months of the intervention for all participants, the following equation was used:
Change in EA (kcal/kg LBM) = EA value at intervention month 6 minus baseline EA value

**Urinary Hormone Measurements**

Samples of first morning urinary voids were collected daily throughout the intervention. Participants were provided with supplies to keep urine samples frozen at home and during transport to the laboratory. All urine samples were corrected for specific gravity using a hand refractometer (NSG Precision Cells) to account for hydration status (162) which has been reported to perform as well as creatinine correction for adjusting urinary hormone concentrations (162). The secretion of E1G and PdG metabolites in the urine parallels serum concentrations of the parent hormones (167). Microtiter plate competitive enzyme immunoassays were used to measure the urinary metabolites E1G and PdG. The E1G (R522-2) and PdG (R13904) assays use a polyclonal capture antibody supplied by Coralie Munro University of California (Davis, CA). The inter-assay coefficients of variation for high and low internal controls for the E1G assay are 12.2% and 14.0% respectively. The PdG intra- and inter-assay variability was determined in-house as 13.6% and 18.7% respectively (60, 64). Urinary LH was determined by coat-a-count immunoradiometric assay (Siemens Healthcare Diagnostics, Deerfield, IL). The sensitivity of the LH assay was 0.15 mIU/ml and the intra-assay and inter-assay coefficients of variation were 1.6% and 7.1%, respectively.

**Serum Hormone Analysis**

An additional measure to document changes in metabolic status included the measurement of circulating triiodothyronine (TT$_3$) concentrations during baseline and after the intervention. We reasoned that changes in EA would be significantly associated
with changes in metabolic status assessed using an objective laboratory measurement, as
this has been previously documented in short term laboratory manipulations of EA (144).
Blood for the measurement of total TT$_3$ was collected after an overnight fast between
0700 and 1000 once during week 3 of baseline and once at the end of baseline for all
participants. The latter two samples were pooled for all baseline hormone analyses. In
addition, blood samples were collected during intervention weeks 5, 9, and 21.
Participants were asked to lie in the supine position for at least 15 minutes after which a
registered nurse or trained phlebotomist obtained a blood sample via venipuncture.
Samples were allowed to clot for at least 30 minutes at room temperature. Samples were
then spun in a centrifuge at 4° Celsius for 15 minutes at 3225.6 g-force (3000 rpm) where
after serum was transferred into appropriately labeled 1.5 mL microtubules and stored at -
80° Celsius until analysis.

Total testosterone (measured to calculate FAI) was measured using a
radioimmunoassay kit (Siemens, Los Angeles, CA) through competitive immunoassay.
The sensitivity of the assay was 0.14 nmol/L (4.0 ng/dl) and the intra-assay and inter-
assay coefficients of variation were 6.4% and 7.5%, respectively. SHBG (measured to
calculate FAI) was assayed using a chemiluminescence analyzer (First Generation
Immulite 1000, Siemens, Deerfield, IL) through competitive immunoassay. The
sensitivity of the assay was 0.2 nmol/L (5.76 ng/dl) and the intra-assay and inter-assay
coefficients of variation were 6.4% and 8.7%, respectively. Serum samples were assayed
for total TT$_3$ using an Immulite chemiluminescent assay (First Generation Immulite 1000,
Siemens, Deerfield, IL). The sensitivity of the assay was 35 ng/dL and the intra-assay
and inter-assay coefficients of variation were 10.3% and 13.3%, respectively. All samples from a given subject were analyzed in duplicate.

**Statistical Analysis**

We calculated sample size based on expected differences and standard deviations from Kopp-Woodroffe et al. (115) who observed resumption of menstrual cyclicity in amenorrheic female athletes who either increased dietary energy intake or decreased exercise energy expenditure, or both, in order to detect meaningful differences in EA. Using an effect size of 4.9 and standard deviation of 2.9, a power coefficient of 0.80 was expected with a sample size of 30 participants with P<0.05 considered to be significant for significant changes within individuals in a group. A power coefficient of 0.80 was expected with a sample size of 3 participants when comparing changes between groups. Data for all variables were tested for outliers and normality using box plot analyses and Shapiro-Wilk tests of normality, respectively. Extreme outliers greater than 3 standard deviations from the mean were not included in our analyses. Independent t-tests were performed to compare differences between groups. Paired t tests were used to test for changes within each group separately. In all analyses, p ≤ 0.05 was considered statistically significant. All data were analyzed using SPSS for Windows (version 18; Chicago, Ill., USA). Data are reported as means ± SEM.

**Results**

**Baseline Characteristics**

Baseline demographic, body composition, and menstrual characteristics for all participants of varying menstrual status are shown in Table 4-1. When grouped
according to the occurrence of menses as the criterion for reproductive recovery, participants in the EAMD-NRM and EAMD-RM groups were similar with respect to age, height, weight, lean body mass, age of menarche, and gynecological age. Both groups exhibited suppressed concentrations of urinary metabolites with little evidence of ovarian activity. The EAMD-RM had a higher BMI (p=0.032), percent body fat (p=0.003), fat mass (p=0.003), and shorter duration of amenorrhea prior to the beginning of the study (p=0.014) than the EAMD-NRM women. When grouped according to the occurrence of menses preceded by an ovulatory cycle as the criterion for reproductive recovery, the EAMD-NRM and EAMD-ROV groups were similar with respect to age, height, weight, BMI, lean body mass, age of menarche, gynecological age, and duration of amenorrhea prior to the beginning of the study. The EAMD-ROV had greater percent body fat (p=0.016) and fat mass (p=0.023) than the EAMD-NRM women. Both of these groups also exhibited suppressed concentrations of urinary metabolites with little evidence of ovarian activity. The modes of purposeful exercise self reported during baseline are represented in Figure 4-3. Running comprised the greatest proportion (28%) of the modes of purposeful exercise when all participants were combined.

The dietary intake, exercise training, and energy availability for all participants of varying menstrual status are shown in Table 4-2. When grouped according to the occurrence of menses as the criterion for reproductive recovery, participants in the EAMD-NRM and EAMD-RM groups were similar with respect to dietary energy and macronutrient intake, $\text{VO}_2\text{peak}$, exercise energy expenditure, exercise volume, exercise frequency, exercise intensity, EA, and TT$_3$ concentrations. However, the EAMD-RM consumed a greater percentage of kilocalories from fat (p=0.042), and had a higher REE
(kcal/kg LBM) (p=0.045), and REE ratio (p=0.053) than the EAMD-NRM women. When grouped according to the occurrence of menses preceded by an ovulatory cycle as the criterion for reproductive recovery, the EAMD-NRM and EAMD-ROV groups were also similar with respect to dietary energy and macronutrient intake, VO$_2$ peak, exercise energy expenditure, exercise volume, exercise frequency, exercise intensity, EA, TT$_3$ concentrations, and REE (kcal/kg LBM). However, the EAMD-ROV had a lower REE ratio (p=0.043) than the EAMD-NRM women.
Table 4-1. Baseline demographic, body composition, and menstrual characteristics of exercising women with EAMD.

<table>
<thead>
<tr>
<th>Demographic characteristics</th>
<th>EAMD-NRM (n=7)</th>
<th>EAMD-RM (n=13)</th>
<th>P-value</th>
<th>EAMD-NRM (n=7)</th>
<th>EAMD-ROV (n=8)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>21.4±1.2</td>
<td>23.9±0.9</td>
<td>0.125</td>
<td>21.4±1.2</td>
<td>25.0±1.1</td>
<td>0.051</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>168.4±2.5</td>
<td>165.7±1.8</td>
<td>0.390</td>
<td>168.4±2.5</td>
<td>164.4±2.3</td>
<td>0.266</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>54.9±3.1</td>
<td>59.1±1.9</td>
<td>0.243</td>
<td>54.9±3.1</td>
<td>56.7±2.7</td>
<td>0.661</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>19.4±1.0</td>
<td>21.5±0.4</td>
<td>0.032*</td>
<td>19.4±1.0</td>
<td>20.9±0.5</td>
<td>0.157</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Body composition characteristics</th>
<th>EAMD-NRM (n=7)</th>
<th>EAMD-RM (n=13)</th>
<th>P-value</th>
<th>EAMD-NRM (n=7)</th>
<th>EAMD-ROV (n=8)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body fat (%)</td>
<td>18.6±2.3</td>
<td>25.8±0.9</td>
<td>0.003*</td>
<td>18.6±2.3</td>
<td>25.3±0.9</td>
<td>0.016†</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>10.2±1.5</td>
<td>15.2±0.8</td>
<td>0.003*</td>
<td>10.2±1.5</td>
<td>14.3±0.8</td>
<td>0.023†</td>
</tr>
<tr>
<td>Lean body mass (kg)</td>
<td>41.8±2.4</td>
<td>41.6±1.3</td>
<td>0.951</td>
<td>41.8±2.4</td>
<td>40.6±1.9</td>
<td>0.693</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Menstrual characteristics</th>
<th>EAMD-NRM (n=7)</th>
<th>EAMD-RM (n=13)</th>
<th>P-value</th>
<th>EAMD-NRM (n=7)</th>
<th>EAMD-ROV (n=8)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of menarche (years)</td>
<td>14.1±0.8</td>
<td>13.5±0.4</td>
<td>0.475</td>
<td>14.1±0.8</td>
<td>13.8±0.4</td>
<td>0.657</td>
</tr>
<tr>
<td>Gynecological age (years)</td>
<td>7.1±1.4</td>
<td>10.2±1.1</td>
<td>0.115</td>
<td>7.1±1.4</td>
<td>11.0±1.5</td>
<td>0.084</td>
</tr>
<tr>
<td>Duration of Amenorrhea (days)</td>
<td>374.1±91.4</td>
<td>155.1±25.7</td>
<td>0.014*</td>
<td>374.1±91.4</td>
<td>167.7±25.8</td>
<td>0.068</td>
</tr>
</tbody>
</table>

Values are reported as mean ± SEM.

EAMD, exercise associated menstrual disturbances
NRM, did not resume reproductive function; RM, resumed menses; ROV, resumed menses preceded by ovulation
*, p<0.05 vs. EAMD-NRM; †, p<0.05 vs. EAMD-NRM
Table 4-2. Baseline dietary intake, exercise training, and energy availability of exercising women with EAMD.

<table>
<thead>
<tr>
<th></th>
<th>EAMD-NRM (n=7)</th>
<th>EAMD-RM (n=13)</th>
<th>P-value</th>
<th>EAMD-NRM (n=7)</th>
<th>EAMD-ROV (n=8)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dietary Intake</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EI (kcal/d)</td>
<td>1779.6±240.6</td>
<td>1871.9±148.6</td>
<td>0.355</td>
<td>1779.6±240.6</td>
<td>1880.2±220.7</td>
<td>0.763</td>
</tr>
<tr>
<td>EI (grams/d)</td>
<td>2643.0±407.9</td>
<td>2997.3±369.4</td>
<td>0.762</td>
<td>2643.0±407.9</td>
<td>3124.8±462.6</td>
<td>0.459</td>
</tr>
<tr>
<td>CHO (% of kcal/d)</td>
<td>61.9±2.7</td>
<td>53.4±2.6</td>
<td>0.055</td>
<td>61.9±2.7</td>
<td>54.2±2.8</td>
<td>0.076</td>
</tr>
<tr>
<td>CHO (g/d)</td>
<td>256.8±29.6</td>
<td>244.6±23.0</td>
<td>0.563</td>
<td>256.8±29.6</td>
<td>256.7±31.8</td>
<td>0.998</td>
</tr>
<tr>
<td>Protein (% of kcal/d)</td>
<td>15.0±1.4</td>
<td>14.3±0.8</td>
<td>0.650</td>
<td>15.0±1.4</td>
<td>13.7±1.1</td>
<td>0.466</td>
</tr>
<tr>
<td>Protein (grams/d)</td>
<td>64.5±11.7</td>
<td>65.5±6.7</td>
<td>0.942</td>
<td>64.5±11.7</td>
<td>65.1±9.7</td>
<td>0.972</td>
</tr>
<tr>
<td>Fat (% of kcal/d)</td>
<td>21.9±2.6</td>
<td>29.0±1.8</td>
<td>0.042*</td>
<td>21.9±2.6</td>
<td>27.3±2.0</td>
<td>0.123</td>
</tr>
<tr>
<td>Fat (grams/d)</td>
<td>43.3±9.1</td>
<td>63.3±6.3</td>
<td>0.093</td>
<td>43.3±9.1</td>
<td>59.8±7.7</td>
<td>0.190</td>
</tr>
<tr>
<td><strong>Exercise training characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VO₂ peak (ml/kg/min)</td>
<td>50.0±2.9</td>
<td>46.5±2.3</td>
<td>0.380</td>
<td>50.0±2.9</td>
<td>47.4±2.4</td>
<td>0.500</td>
</tr>
<tr>
<td>Ex EE (kcal/d)</td>
<td>478.7±101.4</td>
<td>430.3±55.7</td>
<td>0.653</td>
<td>478.7±101.4</td>
<td>397.9±77.5</td>
<td>0.538</td>
</tr>
<tr>
<td>Ex volume (min/wk)</td>
<td>395.3±87.3</td>
<td>347.8±74.1</td>
<td>0.697</td>
<td>395.3±87.3</td>
<td>374.3±117.7</td>
<td>0.891</td>
</tr>
<tr>
<td>Ex frequency (session/wk)</td>
<td>5.0±0.5</td>
<td>4.2±0.4</td>
<td>0.266</td>
<td>5.0±0.5</td>
<td>3.9±0.7</td>
<td>0.232</td>
</tr>
<tr>
<td>Ex intensity (kcal/min)</td>
<td>5.6±1.1</td>
<td>6.7±0.6</td>
<td>0.365</td>
<td>5.6±1.1</td>
<td>6.2±0.9</td>
<td>0.690</td>
</tr>
<tr>
<td><strong>Energy Availability</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EA (kcal/kg LBM)</td>
<td>32.2±5.5</td>
<td>32.9±3.5</td>
<td>0.904</td>
<td>32.2±5.5</td>
<td>33.7±5.4</td>
<td>0.846</td>
</tr>
<tr>
<td>TT₃ (ng/dL)</td>
<td>76.1±6.7</td>
<td>81.2±8.4</td>
<td>0.691</td>
<td>76.1±6.7</td>
<td>81.2±11.5</td>
<td>0.718</td>
</tr>
<tr>
<td>REE (kcal/kg LBM)</td>
<td>26.6±0.8</td>
<td>29.8±1.0</td>
<td>0.045*</td>
<td>26.6±0.8</td>
<td>31.0±1.2</td>
<td>0.011</td>
</tr>
<tr>
<td>REE ratio</td>
<td>0.77±0.04</td>
<td>0.88±0.03</td>
<td>0.053*</td>
<td>0.77±0.04</td>
<td>0.90±0.04</td>
<td>0.043†</td>
</tr>
</tbody>
</table>

Values are reported as mean ± SEM.

EAMD, exercise associated menstrual disturbances
EI, dietary energy intake; Ex, exercise; EE, energy expenditure; CHO, carbohydrate
NRM, did not resume reproductive function; RM, resumed menses; ROV, resumed menses preceded by ovulation

*, p<0.05 vs. EAMD-NRM; †, p<0.05 vs. EAMD-NRM
Figure 4-3. Pie chart representing the modes of purposeful exercise among all participants during the baseline monitoring period.
Changes During the Intervention

Menstrual Status

Changes in daily E1G and PdG from two exercising women with menstrual disturbances at baseline who participated in the intervention are shown in Figure 4-4. In the EAMD-ROV woman (Figure 4-4A), increase in E1G (above 35 ng/ml) and PdG (above 2.5 ug/ml) were observed prior to menses. In the EAMD-NRM woman (Figure 4-4B), resumption of menses was not observed. For the EAMD-RM women, resumption of menses occurred within an average of 64.8±15.1 days (9.7±2.1 weeks). For the EAMD-ROV women, resumption of menses that was preceded by ovulation occurred within an average of 79.2±24.2 days (11.8±3.4 weeks).
Figure 4-4. Reproductive changes from one participant (subject #1 EAMD-ROV) who resumed menses which was preceded by ovulation and one participant (Subject #2 EAMD-NRM) who did not resume menses in response to the intervention. Closed circles represent E1G, estrone-1 glucuronide (ng/ml). Open circles represent PdG, pregnanediol glucuronide (µg/ml). Arrows represent onset of menses.
Body composition and energy status characteristics

The changes in body composition and energy status from baseline to intervention month 6 are presented in Table 4-3. When grouped according to the occurrence of menses as the criterion for reproductive recovery, participants in the EAMD-NRM and EAMD-RM groups were similar with respect to change in body weight, BMI, percent body fat, fat mass, EI, REE (kcal/kg LBM), REE ratio, exercise energy expenditure, exercise volume, exercise frequency, and exercise intensity. A greater change in lean body mass (p=0.027) was observed in the EAMD-RM than EAMD-NRM participants.

When grouped according to the occurrence of menses preceded by an ovulatory cycle as the criterion for reproductive recovery, the EAMD-NRM and EAMD-ROV groups were similar with respect to change in body weight, BMI, fat mass, lean body mass, EI, REE (kcal/kg LBM), REE ratio, exercise energy expenditure, exercise volume, and exercise intensity. A greater change in exercise frequency (p=0.032) was observed in the EAMD-ROV than EAMD-NRM participants.
Table 4-3. Change in body composition, dietary intake and exercise caloric expenditure from baseline to intervention month 6 in exercising women with EAMD.

<table>
<thead>
<tr>
<th>Body Composition Characteristics</th>
<th>EAMD-NRM (n=7)</th>
<th>EAMD-RM (n=13)</th>
<th>P-value</th>
<th>EAMD-NRM (n=7)</th>
<th>EAMD-ROV (n=8)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>0.4±0.7</td>
<td>1.7±0.4</td>
<td>0.093</td>
<td>0.4±0.7</td>
<td>2.2±0.8</td>
<td>0.107</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>0.1±0.2</td>
<td>0.6±0.2</td>
<td>0.138</td>
<td>0.1±0.2</td>
<td>0.9±0.3</td>
<td>0.087</td>
</tr>
<tr>
<td>Fat mass (%)</td>
<td>1.7±0.5</td>
<td>0.9±0.4</td>
<td>0.292</td>
<td>1.7±0.5</td>
<td>1.4±0.7</td>
<td>0.697</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>1.0±0.3</td>
<td>0.9±0.4</td>
<td>0.811</td>
<td>1.0±0.3</td>
<td>1.2±0.6</td>
<td>0.791</td>
</tr>
<tr>
<td>Lean body mass (kg)</td>
<td>-0.6±0.4</td>
<td>0.6±0.3</td>
<td>0.027*</td>
<td>-0.6±0.4</td>
<td>0.5±0.3</td>
<td>0.060</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dietary Intake and Energy Expenditure</th>
</tr>
</thead>
<tbody>
<tr>
<td>EI (kcal/d)</td>
</tr>
<tr>
<td>REE (kcal/kg LBM)</td>
</tr>
<tr>
<td>REE ratio</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Training Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex EE (kcal/d)</td>
</tr>
<tr>
<td>Ex volume (min/wk)</td>
</tr>
<tr>
<td>Ex frequency (session/wk)</td>
</tr>
<tr>
<td>Ex intensity (kcal/min)</td>
</tr>
</tbody>
</table>

Values are reported as mean ± SEM.

NRM, did not resume menses; RM, resumed menses

EI, dietary energy intake; Ex, exercise; EE, energy expenditure; REE, resting energy expenditure

*, p<0.05 vs. EAMD-NRM; †, p<0.05 vs. EAMD-NRM
Energy Availability

The change in EA for both reproductive recovery groups is presented in Figure 4-5. No difference in the change in EA (kcal/kg LBM) from baseline to intervention week 21 was observed between the EAMD-RM and EAMD-NRM women (5.9 ± 4.2 vs. 4.8 ± 4.4 kcal/kg LBM; p=0.867). No difference in the change in EA from baseline to intervention week 21 was observed between the EAMD-ROV than EAMD-NRM women (14.7 ± 10.0 vs. 5.6 ± 5.3 kcal/kg LBM; p=0.452). The proportion of EAMD-NRM and EAMD-RM women with an EA (≥30 kcal/kg LBM) at intervention month 6 was also not significantly different ($\chi^2=0.141$, p=0.658). The proportion of EAMD-NRM and EAMD-ROV women with an EA (≥30 kcal/kg LBM) at intervention month 6 was not significantly different ($\chi^2=0.024$, p=0.876).
Figure 4-5. Bar graphs representing the change in energy availability (kcal/kg LBM) of women with Exercise associated menstrual disturbance (EAMD) who either resumed recovery of menses (EAMD-RM) or did not (EAMD-NRM) (A) and women with EAMD who either resumed recovery of menses which was preceded by ovulation (EAMD-ROV) or did not (EAMD-NRM) (B). Data are expressed as mean ± SEM.
Metabolic Status

The change in TT₃ for both reproductive recovery groups is presented in Figure 4-6. When grouped according to the occurrence of menses as the criterion for reproductive recovery, no difference in the change in TT₃ (ng/dL) from baseline to intervention week 21 was observed between the EAMD-RM and EAMD-NRM women (5.2 ± 3.8 vs. 0.2 ± 4.6 ng/dL, p=0.428). When grouped according to the occurrence of menses preceded by an ovulatory cycle as the criterion for reproductive recovery, no difference in the change in TT₃ (ng/dL) from baseline to intervention week 21 was observed between the EAMD-ROV and EAMD-NRM women (3.9 ± 9.2 vs. -3.8 ± 6.5 ng/dL, p=0.521) were observed.
Figure 4-6. Bar graphs representing the change in T₃ (ng/dL) of women with exercise associated menstrual disturbance (EAMD) who either resumed recovery of menses (EAMD-RM) or did not (EAMD-NRM) (A) and women with EAMD who either resumed recovery of menses which was preceded by ovulation (EAMD-ROV) or did not (EAMD-NRM) (B). Data are expressed as mean ± SEM.
Discussion

This was the first study to examine whether trained women with EAMD who displayed reproductive recovery experienced a greater increase in EA when compared to trained premenopausal women with EAMD who did not display reproductive recovery. We also tested whether a greater proportion of women who displayed reproductive recovery exhibited an EA ($\geq 30$ kcal/kg LBM) when compared to those who did not display reproductive recovery. Our results indicate that women with EAMD who displayed reproductive recovery, whether defined as the resumption of menstrual bleeding or resumption of menstrual bleeding preceded by ovulation, did not experience a greater increase in EA when compared to women with EAMD who did not display reproductive recovery. Our findings can be compared to those of Kopp-Woodroffe et al. (115) who examined the effects of a successful 20 week diet and training intervention on the menstrual status of 4 female amenorrheic athletes. The latter intervention included supplementing EI with one sport nutritional supplement daily and reducing training from 7 to 6 days a week. Although the magnitude of change in EA we observed is similar to the findings of Kopp-Woodroffe et al. (115), when a control group who did not display reproductive recovery is used for comparison, no association between a change in EA and a change in ovarian activity can be discerned.

Because we found no association between the change in EA and the change in reproductive function, it is not surprising that the proportion of women who displayed reproductive recovery with an EA ($\geq 30$ kcal/kg LBM) was not significantly different from the women who did not display reproductive recovery. Our results are in contrast to Loucks et al. (144) who demonstrated that significant reductions in reproductive and
metabolic hormones and unfavorable alterations in bone turnover makers occur below an EA of 30 kcal/kg LBM. Differences in our results may be explained by several factors. The manipulations of EA in Loucks et al. (144) were performed under controlled laboratory conditions where EI was provided with a clinical dietary product and a target level of EEE was monitored with indirect calorimetry. The relevance of EA and or a threshold of EA which defines reproductive recovery have not been tested in a field setting where more conventional methods available to exercising women such as diet logs, exercise logs, and or heart rate monitoring have been used to assess EA (EI-EEE/kg LBM). As such, the variability in the components of EA may have contributed to the lack of observed differences in the proportion of women who displayed an EA (≥30 kcal/kg LBM) at intervention month 6. Day to day variability in EI has been reported to range from approximately 1 to 33% in females (181, 227). When compared to doubly labeled water, heart rate monitoring has been shown to estimate energy expenditure within -4.0 to 11.4% in females (132) and 7 day physical activity records which use the Ainsworth et al. (5) compendium to estimate the energy cost of movements have been shown to overestimate total energy expenditure by 7.9±3.2 (47). The inaccuracies in these methods may therefore have resulted in EA values that do not accurately reflect the same energy status as the 30 kcal/kg LBM EA condition of Loucks et al. (137, 144).

In addition to the lack of changes in EA, we did not observe a greater change with reproductive recovery in another index of energy status, circulating TT₃ concentrations. Changes in circulating TT3 have been associated with the induction and recovery of reproductive function in exercising monkeys (258). Our TT₃ findings contrast those of Williams et al. (258) who observed significant increases in total TT₃ concentrations
concomitant with the resumption of menses in amenorrheic cynomologus monkeys who increased EI while maintaining strenuous exercising training. Gagneux and colleagues (81) documented lower total and free TT$_3$ concentrations in humans than chimpanzees (81) and also suggested that the down-regulation of thyroid-stimulating hormone could be at a different set point in chimpanzees than humans. If so, it is plausible that the set-point at which changes in thyroid hormones occur and the magnitude of these changes could be greater for primates which might help to account for the lack of significant change in our TT$_3$ concentrations when compared to those of Williams et al. (258). It is also possible that the small sample size in combination with the variability of our TT$_3$ measurements contributed to the lack of observed differences in TT$_3$ concentrations.

In contrast however, our TT$_3$ findings are more similar to those of Loucks et al. (141) who found similar total TT$_3$ concentrations between amenorrheic and eumenorrheic athletes. Moreover, Arimura et al. (8) observed similar TT$_3$ concentrations between eumenorrheic and amenorrheic women with anorexia nervosa, and De Souza et al. (57) who observed similar TT$_3$ concentrations between exercising women with subtle and severe menstrual disturbances. Although circulating TT3 did not differ, these studies did report other signs of energy deficiency that were significantly associated with severe menstrual disturbances such as increased cortisol concentrations (8), lower REE (kcal/kg LBM) (58), and increased ghrelin concentrations (59). Further studies are required to determine if other indicators of energy status might confirm positive changes in energy balance that would be associated with changes in menstrual status. For example, significant changes in body weight have been observed in monkeys who resumed menses
in response to an increase in EI while maintaining a strenuous exercise training program (258).

Although we observed changes in a positive direction for body weight and body composition, it could be argued that the lack of significant change in these variables would not be surprising. These findings are similar to cross sectional studies (139, 141) that have reported no differences in body weight, BMI, or percent body between eumenorrheic and amenorrheic athletes (141). Similarly, Williams et al. (258) did not observe changes in body weight in cynomolgus monkeys who developed amenorrhea in response to a gradual strenuous exercise training regimen. While large changes in body weight are generally concomitant with alterations in reproductive function resulting from changes in EI and or energy expenditure, changes in energy status have also been documented with objective laboratory measures when body weight remains the same (257, 259).

It is possible that subtle changes in energy status were not captured using the variables assessed in the current study. Other indices of energy status including ghrelin (59), PYY (205-206), and even changes in the parameters of energy balance such as non-exercise associated thermogenesis (NEAT) (129) might prove to be useful measures in assessing subtle changes in energy status that are not captured in the EA variable. In humans, fasting ghrelin concentrations have been shown to be negatively correlated with 24 hour EI (219). In particular, ghrelin concentrations decrease following EI with the decrease being related to the amount of calories ingested (127). Several studies have reported higher ghrelin concentrations in exercising women with amenorrhea (58-59) and adolescent athletes with anorexia nervosa (1, 38). It has been suggested that elevated
ghrelin concentrations may be related to the suppression of reproductive function in amenorrheic women (205). In fact, ghrelin has been shown to suppress the secretion of LH and follicle stimulating hormone in young women (109). Similarly, elevated PYY concentrations have been observed in energy deficit exercising women (206). Last, NEAT is a component of energy balance that is not accounted for in the calculation of EA. NEAT can vary by as much as 2000 kcal/d and both increases and decreases in NEAT have been detected during states of state of positive and negative energy balance, respectively (125, 129, 199). If changes in NEAT were to occur specifically in association with reproductive suppression, then using EA to identify menstrual disturbances would be unsuccessful. Taken together, the above mentioned indices may prove to be more useful in assessing energy and reproductive status than EA alone. Furthermore, because changes in energy balance are associated with a complex interplay of changes in metabolism, endocrine, and neuroendocrine factors, it may be that a combination of variables may prove to be the best predictive index of impending changes in menstrual function.

It is also possible that psychological stress (18, 184, 255) may account for some proportion of the observed EAMD suggesting that EA (EI-EEE/kg LBM) may not have played a role in the menstrual disturbances observed in some of our women. Likewise, it is possible that changes in psychological stress or the perception of life stressors could have accounted for some degree of reproductive recovery we observed. In support, studies have shown that cognitive behavioral therapy (17) and hypnotherapy (238) can be an effective treatment for functional hypothalamic amenorrhea. In the current study, a registered dietician met with the participants throughout the intervention to review their
diet and provide strategies to meet their target calorie intake. A clinical psychologist or licensed clinical social worker met with the participants throughout the intervention to monitor their general psychological health and provide assistance in helping to implement lifestyle changes that participants decided to make in the context of the intervention. Several of the participants in this study were in the control group of the intervention. These participants also met with the nutritionist, clinical psychologist, and or licensed clinical social worker; however, the purpose of these visits was to monitor compliance as a control participant in the investigation. Williams et al. (258) showed that low-level stressors which alone have little impact on the reproductive axis can act in a synergistic fashion with metabolic stressors i.e. moderate energy deficit to compromise reproductive function. It is therefore possible that the emotional support indirectly provided by the nutritionist, psychologist, and or social worker may have alleviated some degree of psychological stress which helped to play a role in the reproductive recovery of some of our participants. This provides a plausible explanation for the lack of increased EA in our exercising women who displayed reproductive recovery.

It is also possible that baseline differences in body composition or duration of amenorrhea may have played a role in the recovery of reproductive function. Perhaps greater fat mass conferred an advantage to those exercising women who displayed reproductive recovery. In particular, leptin is secreted from adipocytes and circulates at concentrations highly correlated to the degree of adiposity (180). This adipokine relays information regarding nutritional status to the hypothalamus (10) and its administration has been shown to be an effective pharmacological treatment for hypothalamic amenorrhea (37). Therefore, it is possible that if increased body fat and thus leptin
concentrations are present, the degree of change in energy status needed to stimulate reproductive recovery may be smaller. Future studies are needed to examine the changes in EA that occur with concomitant changes in leptin with respect to the recovery of menstrual function. Lastly, the duration of amenorrhea was shorter at baseline in our exercising women who resumed menses. Perhaps chronic exposure to low EA conditions and resulting severe menstrual disturbances confers a disadvantage to the recovery of reproductive function. Future studies are needed to assess the role of duration of amenorrhea in the recovery of reproductive function.

A notable strength and novel feature of this study is the extent to which reproductive recovery was assessed. We used clinical and physiological criteria to define reproductive recovery in two ways representing different levels of “robustness” of reproductive recovery. The first being the resumption of menstrual bleeding and the second as the resumption of menstrual bleeding preceded by ovulation. Limitations of this study include a small sample size, however our study still represents the only one to date that examines the recovery of menstrual function in exercising women using a control group for comparison. A strength of this study includes the relatively careful measurements of EA, in that we took great care to achieve the highest degree of accuracy possible using several non-laboratory based measures that rely heavily on self report. It is important to consider though that several studies have shown inaccuracies when measuring energy intakes using self-reported food records, particularly related to under reporting (203). However, all of the participants were trained to accurately record food intake, provided with a foods amount packet which contained diagrams illustrating container sizes, cuts of meat, and various circles and squares to estimate portion sizes,
and provided with a completed diet record as a reference. As well, the reporting of physical activity can vary greatly depending on participant compliance (172).

**Conclusion**

This study was the first to show that trained women with EAMD who displayed reproductive recovery during an intervention to improved energy status, whether defined as the resumption of menstrual bleeding or the resumption of menstrual bleeding preceded by ovulation, did not experience a greater increase in EA when compared women with EAMD who did not display reproductive recovery. In addition, the proportion of women with EAMD who displayed reproductive recovery with an EA $\geq 30$ kcal/kg LBM in response to the intervention was not different when compared to the women with EAMD that did not display reproductive recovery. Similar to the lack of observed changes in EA, no significant changes in TT$_3$ concentrations, body weight, BMI, EI, EEE, REE, or REE ratio were observed in response to the intervention even when a more robust definition of reproductive recovery was used. Future studies with a larger sample size and more accurate indices of EA or other energy parameters are needed to confirm the role of changes in dietary intake and energy status in the recovery of reproductive function and whether this variable is a useful tool for monitoring reproductive status. Moreover, the possibility that other factors such as changes in psychological stress may play a role in the recovery of reproductive function must be more closely examined.
Chapter 6

The prediction of energy deficiency in exercising women with menstrual disturbances by various indices of metabolic status

Introduction

Exercising women who restrict their dietary energy intake (EI), exercise for long periods of time, and limit their food choices may become energy deficient and consequently develop menstrual disturbances (32, 171, 188). These eating and exercise behaviors are often a result of the internal and external pressures placed on exercising women to maintain low body weight or lean physique in order to improve performance (14). Several negative clinical outcomes such lower bone mineral density (66), altered vascular function (177), higher injury rates (182), disordered eating (84, 241), reductions in resting energy expenditure (REE) (168), and suppressed metabolic hormones (58) have all been documented in energy deficient exercising women with menstrual disturbances. Prolonged energy deficiency and the suppression of menstrual cyclicity alone, or in combination, can lead to bone loss (66). The Female Athlete Triad, a syndrome comprising three of the above outcomes, i.e., low energy availability (EA), amenorrhea, and osteoporosis is a widespread concern among the sports medicine community and recent research efforts have been directed to translational solutions to prevent and treat chronic energy deficiency in order to offset these negative sequelae (171).

Other than a recent report in high school athletes (98), no studies to date have examined the prevalence of an energy deficiency in a large population of exercising women. To quantify energy balance (EB), both EI and total daily energy expenditure
(TDEE) must be estimated (EB kcal/d = EI - TDEE) (55). Self reported diet logs are a common method used to estimate EI (234). A number of techniques such as indirect calorimetry (2), heart rate monitors (36), accelerometers (226), and exercise logs (112) can be used to estimate TDEE or its components which include REE, non-exercise associated thermogenesis (NEAT), exercise energy expenditure (EEE), and thermic effect of food (TEF). The high cost and or length of time required to estimate EI and TDEE with the above methods is not however feasible or practical for most exercising women (209). With respect to menstrual status, De Souza and colleagues (64) showed that almost half of exercising women experience subtle menstrual disturbances and one third might be amenorrheic depending on their sport. In addition to the difficulties with estimating EB, characterizing subtle endocrine abnormalities such as luteal phase defects and anovulation (68) is difficult as these reproductive disturbances must be detected with repeated hormonal measurements (62, 64) or careful monitoring of basal body temperatures (242). A number of limitations therefore exist for exercising women to assess their energy and menstrual status.

Several indices of energy status such as energy availability (EA), REE, REE ratio (actual to predicted), metabolic hormones, and body composition have been used to corroborate an energy deficiency, adaptations to conserve energy, and the risk of menstrual disturbances (58, 84, 144). In addition, NCAA Division I pre participation questionnaires have been used to screen for components of the Female Athlete Triad and include questions addressing energy status such as “do you lose weight regularly to meet weight requirements for your sport?” (157). Despite the number of methodologies available to assess energy and menstrual status, no studies to date have examined which
of these methods best predicts an energy deficiency or menstrual disturbance in exercising premenopausal women. This is an important question to address as female military recruits (120), exercising premenopausal women (42, 64, 98, 196), and high school female athletes (98, 174, 194) are all susceptible to menstrual disturbances which occur secondary to an energy deficiency (32, 256, 258, 260). The identification of a method that best predicts an energy deficiency or menstrual disturbance may provide a way for exercising women to assess their energy and menstrual status without the need for measuring all the components of EB or providing repeated biological specimens for hormonal measures. In addition, it may help to inform clinicians, athletic trainers, nutritionists, and coaches of the best tool to identify exercising women at risk for the Female Athlete Triad.

The purpose of this study was twofold: 1) to examine which of several indices of energy status best predicts the presence of an energy deficit and 2) to examine which of several indices of energy status best predicts menstrual status (ovulatory vs. exercise associated menstrual disturbances) in premenopausal exercising women.

Methods

Experimental Design

We combined data from two studies performed at the University of Toronto and The Pennsylvania State University. Both studies were designed to examine menstrual disturbances and alterations in energy balance in premenopausal women. The current study includes data from 91 exercising women in whom assessments of menstrual and energy status were performed. Measurements of the variables of interest, i.e., menstrual
status, body composition, dietary energy intake, exercise energy expenditure, resting energy expenditure, total triiodothyronine and ghrelin concentrations were conducted by the same investigators at both sites using similar if not identical methods.

To examine which indices of energy status best predicts an energy deficit using logistic regression analyses, we grouped our exercising women into one of the following two categories: 1) energy deficient if participant exhibited a difference of more than -500 kcal/d between EI and TDEE, or 2) energy replete if participant exhibited a difference less than -500 kcal/d between EI and TDEE. The variables used to test which indices of energy status best predicts an energy deficit included: EA, operationally defined as dietary energy intake (EI) minus exercise energy expenditure (EEE) relative to kilograms of lean body mass (LBM) (EA=EI-EEE/kg LBM), REE ratio, REE (kcal/kg LBM), total T3 (ng/dL), ghrelin (pg/mL), body fat (%), and body mass index (kg/m^2). To examine which indices of energy status best predicts menstrual status using logistic regression analyses, we tested how well these same variables predicted whether our subjects were 1) ovulatory, or 2) exhibited an exercise associated menstrual disturbance.

Participants

Participants were recruited by fliers posted on campus and in the surrounding community, newspaper advertisements, and classroom announcements targeting exercising women for a study on women’s health. Initial eligibility criteria included: 1) no history of or current serious medical conditions, 2) no current clinical diagnosis of an eating or psychiatric disorder based on self-report or an interview with a clinical psychologist or licensed clinical social worker, 3) age 18-35 years, 4) non-smoking, 5) no medication use that would alter metabolic or reproductive hormone concentrations, 6) ≥ 2
hrs/wk of purposeful exercise; 7) no history of or clinical diagnosis of polycystic ovarian syndrome (PCOS) and/or a free androgen index (FAI), calculated as (total testosterone (nmol/L) / sex hormone binging globulin (SHBG) (nmol/L))*100) (200), > 6 in participants in which these hormones were measured. An FAI greater than 6.0 have been reported to be consistent with hyperandrogenemia (88, 198) and represents values greater than three standard deviations in our reference population which consisted of healthy premenopausal exercising women with documented ovulatory menstrual status (61, 64).

**Screening Procedures**

During an initial visit, participants were informed of the purpose, procedures, and potential risks of participation in the study before signing an informed consent approved by either the Human Ethics Board at the University of Toronto or the Institutional Review Board at The Pennsylvania State University. Once consent was obtained, height and weight were measured, and participants completed questionnaires to assess demographics, medical history, exercise history, menstrual history, eating behaviors, bone health, and mental health. A physical exam was performed on most participants by an on-site clinician to determine overall health and check for physical symptoms of PCOS such as acne or hirsutism. In addition, in most participants a fasting blood draw was analyzed for a complete blood count, basic chemistry panel, and an endocrine panel which included measures of LH, follicle stimulating hormone, thyroid stimulating hormone, thyroxine, prolactin, dihydroepiandrosterone (Quest Diagnostics, Pittsburgh, PA), total testosterone, and SHBG to rule out illness or endocrine or metabolic disease for most participants. Participants met with a General Clinical Research Center (GCRC) registered dietitian or trained laboratory personnel to receive instructions on how to
complete 3-day diet logs (2 weekdays and 1 weekend day). Additionally, dual-energy x-ray absorptiometry (DXA) scans of the total body, lumbar spine, and dual femur were performed to assess bone mineral density (BMD) and body composition.

**Aerobic Capacity**

Peak aerobic capacity (VO\(_2\) peak) was measured on a treadmill by indirect calorimetry using an on-line MedGraphics Modular VO\(_2\) System (St Paul, MN) or SensorMedics Vmax metabolic cart (Yorba Linda, Calif., USA) during baseline using methods that have previously been published (58).

**Anthropometrics**

Total body weight was measured by a digital scale each week for 4 weeks in the laboratory to the nearest 0.01 kg wearing t-shirt and gym shorts. The mean of these measurements in presented. BMI was calculated as a ratio of weight to height (kg/m\(^2\)). Height was measured to the nearest 1.0 cm without shoes. Body composition, including percent body fat, fat mass (FM), fat free mass (FFM), and LBM was analyzed by a certified technician using DXA on one of three machines. The majority of participants were scanned on either a GE Lunar Prodigy DXA scanner (n=57) (GE Lunar Corporation, Madison, WI, enCORE 2002 software, version 6.50.069) or a GE Lunar iDXA scanner (n=26) (General Electric Lunar Corporation, Madison, WI, enCORE 2008 software version 12.10.113). Remaining participants were scanned on a Hologic QDR4500 DXA scanner (n=8) (Hologic Inc., Bedford, MA). Consistent with the International Society of Clinical Densitometry guidelines, cross calibration studies were performed to remove systematic bias between the systems. For the cross calibration study between the Lunar Prodigy and Lunar iDXA, fourteen participants were scanned in
triplicate on both machines. The majority (n=8) were scanned on both machines within 5 days while approximately one month lapsed between scans for some participants (n=6). The values for body composition obtained on each scanner were found to be highly correlated with no significant difference between the population mean values. For the cross calibration study between the Hologic QDR4500W and the Lunar iDXA, thirty-two participants were scanned in duplicate on both machines on the same day. Equations were derived using simple linear regression to remove biases, and body composition values obtained from both the Lunar Prodigy and the Hologic QDR-4500W were calibrated to the Lunar iDXA.

**Dietary Energy Intake**

Measures of EI were calculated from 3-day diet logs completed during the study period. Participants were provided with a food scale (ECKO Kitchen Scale) and/or food amounts packet. The packet contained diagrams illustrating container sizes, cuts of meat, and various circles and squares which are typically used when estimating portion size for foods like bowls of cereal. Participants were encouraged to use these scaled diagrams as a guide for describing dimensions and sizes. Also, included in the packet was a sample page of an accurately completed diet record provided as a reference. Participants were asked to record all foods and beverages consumed on 2 weekdays and 1 weekend day. Registered dietitians and/or trained laboratory personnel instructed each subject on how to record EI and then later reviewed diet logs with participants for completeness and accuracy. The nutrient data from the 3-day diet logs were coded and analyzed using Nutritionist Pro (Version 3.1, Axxya Systems, Stafford, TX) or the Nutrition Data System for Research (NDSR 2008 Version; University of Minnesota; Minneapolis, MN).
Resting Energy Expenditure

REE was measured by indirect calorimetry using a Sensormedics Vmax metabolic cart (Yorba Linda, CA) using methods that have previously been published (196). We compared laboratory assessed REE with a predicted REE (pREE) using the Harris-Benedict equation to estimate how much each individual’s measured REE deviated from the pREE. Our lab has previously shown that energy deficient exercising women exhibited a lower measured to predicted REE (57, 66, 84).

Non-exercise Associated Thermogenesis

NEAT was obtained from a reference population of exercising women (n=61) of similar age (18-27 years), BMI (17-28 kg/m²), and identical geographic location (northeastern United States) who were recruited for a study on women’s health. Participants wore a tri-axial RT3 research activity monitor (Stayhealthy, Monrovia, CA) for a 7 day period. The average kilocalories/d measured by the RT3 research activity monitors during this 7 day period was used to calculate NEAT.

Exercise Energy Expenditure

Participants completed exercise logs where all purposeful exercise sessions greater than 10 minutes in duration with a heart rate above 90 beats per minute were recorded for a 7 day period. Purposeful exercise included activities such as elliptical, pilates, running, or strength training, but not daily living activities such as house cleaning or walking a dog. Energy expended during these purposeful exercise sessions was measured using the OwnCal feature of the Polar S610 or RS400 heart rate monitors (Polar Electro Oy, Kempele, Finland) (49). The OwnCal feature has been validated for the use in calculating exercise energy expenditure from heart rate (95-96, 107). This
feature uses body weight, height, age, gender, VO\textsubscript{2peak}, individual maximum heart rate, individual heart rate in a sitting position, and heart rate during exercise to derive kilocalories from energy expenditure. Actual VO\textsubscript{2peak} values were input into the heart rate monitors to compute exercise energy expenditure. The Polar S601 and RS400 heart rate monitors include rest in their estimation of energy expenditure. To estimate only EEE, we subtracted measured REE (kilocalories/min) from the Polar heart rate monitors estimation of energy expenditure. For purposeful exercise sessions in which participants did not wear the heart rate monitors, the Ainsworth et al. (4, 6) compendiums of physical activities were used to determine the appropriate metabolic equivalent (MET) level for the exercise performed (3). To calculate the energy expended during the exercise session, the MET value includes a resting component. To estimate only EEE, we therefore subtracted measured REE (kilocalories/min) from this value. Determination of MET levels from exercise logs from both experimental sites was made by the same research team.

**Energy Balance**

Energy balance was calculated as EI minus REE, NEAT, EEE, and thermic effect of food (TEF) (EB kcal/d = EI – (REE+NEAT+EEE+TEF)) using the data described above for EI, REE, NEAT, and EEE. Ten percent of the energy associated with REE, NEAT, and EEE was used to estimate TEF (TEF kcal/d = 0.10*(REE+NEAT+EEE)). We operationally defined participants as energy deficient if they exhibited a ≥ 500 kcal/d difference between EI and TDEE as several studies have shown low EA, menstrual disturbances, and suppressed metabolic rate and hormones below a deficit of this value.
We therefore operationally defined participants as energy replete if they exhibited a $\leq -500$ kcal/d difference between EI and TDEE.

**Energy Availability**

EA was operationally defined as EI minus EEE relative to kilograms of LBM (EA = (EI – EEE)/LBM) and calculated using the data described above for EI, EEE, and LBM. We calculated EA using the averages of each participant’s values for EI, EEE on workout days, and the LBM from the DXA scan.

**Menstrual Status**

The classification of menstrual status was based on self-reported menstrual histories, menstrual calendars used to chart menstrual symptoms i.e., cramps, bleeding, spotting, discharge, etc., and daily measurements of urinary estrone-1-glucuronide (E1G), pregnanediol glucuronide (PdG), and mid-cycle LH profiles. Participants who self-reported eumenorrheic menstrual status, defined as regular menstrual cycle intervals of 26-35 days, were monitored for 1-3 menstrual cycles. Participants who self-reported no menses within the past 3 months or 6 or fewer menses within the past year collected first morning urine samples beginning on an arbitrary day for 28 days.

Ovulatory status was confirmed by the presence of a urinary LH peak, identified as a peak concentration above 25 mIU/ml occurring after a mid-cycle E1G peak greater than 35 ng/ml, and followed by a peak luteal phase PdG concentration above 5 µg/ml in participants who exhibited menstrual cycles of 26-35 days. Luteal phase defects were confirmed when the luteal phase was either less than 10 days (short) or when the sum of the 3 day mid luteal peak PdG (sum of mid luteal peak PdG ± 1 day) was less than 10 µg/ml and when the PdG peak concentration was below 5 µg/ml but greater than 2.5
μg/ml in participants who exhibited menstrual cycles of 26-35 days (inadequate) (64). Anovulatory cycles were confirmed as cycles in which a minimal increase in EIG was observed concomitantly with a failure of LH to rise at midcycle and when a luteal phase exhibited no increase in PdG concentration above 2.5 μg/ml in participants who exhibited menstrual cycles of 26-35 days (64). Oligomenorrhea was confirmed if menses occurred at intervals of 36-90 days and if participants self-reported 6 or less menstrual cycles in the last year prior to the study. Lastly, functional hypothalamic amenorrhea was assessed by confirming a negative pregnancy test, no menses in the past 90 days, and chronically suppressed E1G and PdG profiles (64). After consideration of menstrual status, participants were grouped into one of the following two categories: 1) ovulatory (Ov, n=20), with consistently ovulatory cycles for the duration of the menstrual cycle collection period, 2) exercise associated menstrual disturbances (EAMD, n=71) which consisted of inconsistent presentations of subtle menstrual disturbances including various inconsistent combinations of ovulatory, luteal phase defects, and anovulatory cycles from cycle to cycle for the duration of the menstrual cycle collection period, consistent anovulatory menstrual cycles for the duration of the menstrual cycle collection period, oligomenorrheic menstrual cycle (36-90 days), and amenorrhea with no menses for the duration of the collection period and at least 3 months before the study.

**Urinary Hormone Measurements**

All urine samples were corrected for specific gravity using a hand refractometer (NSG Precision Cells) to account for hydration status (162) which has been reported to perform as well as creatinine correction for adjusting urinary hormone concentrations (162). Microtiter plate competitive enzyme immunoassays were used to measure the
daily values for urinary metabolites E1G and PdG as previously reported (60, 64). Urinary LH was measured in samples during the ovulatory phase of the menstrual cycle as determined by visual confirmation of a pre-ovulatory rise in E1G followed by a sustained increase in PdG. LH was determined using a coat-a-count immunoradiometric assay (Siemens Healthcare Diagnostics, Deerfield, IL). The sensitivity of the LH assay was 0.15 mIU/ml and the intra-assay and inter-assay coefficients of variation were 1.6% and 7.1%, respectively.

**Serum Measurements**

Blood was collected after an overnight fast before 1000 hr once during the study period. Participants were asked to lie supine for at least 15 minutes after which a blood sample was obtained via venipuncture. Samples were allowed to clot for at least 30 minutes at room temperature and then spun in a centrifuge at 4°Celsius for 15 minutes at 3000 rpm whereafter serum was transferred into appropriately labeled 1.5 mL microtubules and stored at -80°Celsius until analysis. Serum samples were assayed in duplicate for total triiodothyronine (TT₃) using an Immulite chemiluminescent assay (First Generation Immulite 1000, Siemens, Deerfield, IL). The sensitivity of the assay was 35 ng/dL and the intra-assay and inter-assay coefficients of variation were 10.3% and 13.3%, respectively. Total ghrelin was measured an RIA for total ghrelin (Linco Research, St. Charles, MO). The sensitivity of the assay was 93 pg/ml and the intra-assay and inter-assay coefficients of variation were 2.0 and 15.7%, respectively. To determine FAI for screening purposes, total testosterone was measured using a radioimmunoassay kit (Siemens, Los Angeles, CA) through competitive immunoassay. The sensitivity of the assay was 0.14 nmol/L (4.0 ng/dl) and the intra-assay and inter-
assay coefficients of variation were 6.4% and 7.5%, respectively. SHBG was assayed in duplicate using a chemiluminescence analyzer (First Generation Immulite 1000, Siemens, Deerfield, IL) through competitive immunoassay. The sensitivity of the assay was 0.2 nmol/L (5.76 ng/dl) and the intra-assay and inter-assay coefficients of variation were 6.4% and 8.7%, respectively.

**Statistical Analysis**

All variables were tested for outliers and normality using box plot analyses and Kolmogorov-Smirnova tests of normality, respectively. Extreme outliers were not included in our analyses. Cook’s distance was performed to measure the overall influence of cases on a model. No influential cases with a value greater than 1 were identified. Multicollinearity analyses were performed to examine the relationship among predictor variables. Variance inflation factors were all below 10. Logistic regression analyses using the Enter method were performed to examine individual predictors of energy (energy deficient or energy replete) and menstrual status (ovulatory or exercise associated menstrual disturbance). Stepwise regression analyses were then performed to examine the set of variables that best predicts an energy deficit or having a menstrual disturbance. Independent t-tests were performed to compare differences between groups. Data are reported as means ± SEM, and p ≤ 0.05 was considered statistically significant. All data were analyzed using SPSS for Windows (version 18; Chicago, Ill., USA).
Results

Demographic, anthropometric, body composition and menstrual status characteristics

Demographic, anthropometric, body composition and menstrual status characteristics of our exercising women with varying menstrual and energy status are presented in Table 5-1. When grouped according to menstrual status, the OV and EAMD women were similar (p>0.05) with respect to height, weight, BMI, body fat (%), fat mass, and lean body mass. The EAMD women were younger (p=0.043), had a later age of menarche (p=0.027), and a younger gynecological age (p=0.006) than the OV women. When grouped according to energy status, the ER and ED women were similar (p>0.05) with respect to height, weight, BMI, body fat (%), fat mass, lean body mass, and age of menarche. The ED women were younger (p=0.036) and had a younger gynecological age (p=0.013) than the ER women.
Table 5-1. Demographic, anthropometric, body composition and menstrual characteristics of exercising women with varying energy and menstrual status.

<table>
<thead>
<tr>
<th></th>
<th>ED</th>
<th>ER</th>
<th>P-value</th>
<th>EAMD</th>
<th>OV</th>
<th>P-value</th>
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<td><strong>Demographic characteristics</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>22.2±0.5</td>
<td>24.1±0.8</td>
<td>0.036†</td>
<td>22.6±0.5</td>
<td>24.9±1.1</td>
<td>0.043*</td>
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<td>Height (cm)</td>
<td>166.4±0.9</td>
<td>164.7±0.8</td>
<td>0.155</td>
<td>165.9±0.7</td>
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<td>0.290</td>
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<tr>
<td>Weight (kg)</td>
<td>59.0±1.0</td>
<td>57.0±0.8</td>
<td>0.129</td>
<td>58.1±0.8</td>
<td>57.8±1.3</td>
<td>0.831</td>
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<tr>
<td>Body mass index (kg/m²)</td>
<td>21.3±0.3</td>
<td>21.0±0.3</td>
<td>0.561</td>
<td>21.1±0.2</td>
<td>21.4±0.4</td>
<td>0.607</td>
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<td><strong>Body composition characteristics</strong></td>
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<td>Body fat (%)</td>
<td>25.8±0.6</td>
<td>24.8±0.8</td>
<td>0.329</td>
<td>25.4±0.6</td>
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<td>Fat mass (kg)</td>
<td>15.1±0.5</td>
<td>14.2±0.5</td>
<td>0.216</td>
<td>14.7±0.4</td>
<td>14.5±0.6</td>
<td>0.836</td>
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<td>Lean body mass (kg)</td>
<td>41.8±0.7</td>
<td>41.2±0.6</td>
<td>0.526</td>
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<tr>
<td>Age of menarche (years)</td>
<td>13.0±0.2</td>
<td>12.7±0.2</td>
<td>0.299</td>
<td>13.1±0.3</td>
<td>12.2±0.3</td>
<td>0.027*</td>
</tr>
<tr>
<td>Gynecological age (years)</td>
<td>8.9±0.5</td>
<td>11.4±0.8</td>
<td>0.013†</td>
<td>9.4±0.5</td>
<td>12.7±1.1</td>
<td>0.006*</td>
</tr>
</tbody>
</table>

Values are reported as mean ± SEM.

OV, ovulatory; EAMD, exercise associated menstrual disturbances

EB, energy replete (≤-500 kcal/d); ED, energy deficient (≥-500 kcal/d)

*, p<0.05 vs. OV; †, p<0.05 vs. EB
Dietary intake, training characteristics, and metabolic status

Dietary intake, training characteristics, and energy status of our exercising women with varying menstrual and energy status are presented in Table 5-2. When grouped according to menstrual status, the OV and EAMD women were similar (p>0.05) with respect to energy (kcal/d) and carbohydrate (% of kcal/d), protein (% of kcal/d), and fat (% of kcal/d) intake, VO$_{2}$peak, exercise energy expenditure, exercise volume, exercise frequency, exercise intensity, EB, EA, and TT$_3$ and ghrelin concentrations. The EAMD women had a lower REE (kcal/kg LBM) (p=0.001) and REE ratio (p=0.001) than the OV women. When grouped according to energy status, the ED and ER women were similar (p>0.05) with respect to carbohydrate (% of kcal/d), protein (% of kcal/d), and fat (% of kcal/d) intake, VO$_{2}$peak, exercise energy expenditure, exercise volume, exercise frequency, exercise intensity, REE ratio, and TT$_3$ and ghrelin concentrations. The ED women consumed less energy (kcal/d) (p<0.001) and exhibited a lower EB (p<0.001), EA (p<0.001), and REE (kcal/kg LBM) (p=0.017) than the ER women.
Table 5-2. Dietary intake, training characteristics, and energy status of exercising women with varying energy and menstrual status.

<table>
<thead>
<tr>
<th></th>
<th>ED (n=47)</th>
<th>ER (n=44)</th>
<th>P-value</th>
<th>EAMD (n=51)</th>
<th>OV (n=20)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dietary Intake</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EI (kcal/d)</td>
<td>1535.0±69.7</td>
<td>2195.6±55.7</td>
<td>&lt;0.001†</td>
<td>1825.4±65.1</td>
<td>1957.4±114.5</td>
<td>0.338</td>
</tr>
<tr>
<td>CHO (% of kcal/d)</td>
<td>57.4±1.7</td>
<td>54.3±1.6</td>
<td>0.184</td>
<td>56.3±1.3</td>
<td>54.5±2.7</td>
<td>0.529</td>
</tr>
<tr>
<td>Protein (% of kcal/d)</td>
<td>16.2±0.7</td>
<td>15.1±0.7</td>
<td>0.307</td>
<td>15.7±0.6</td>
<td>15.4±1.1</td>
<td>0.800</td>
</tr>
<tr>
<td>Fat (% of kcal/d)</td>
<td>24.9±1.4</td>
<td>28.3±1.3</td>
<td>0.078</td>
<td>25.7±1.0</td>
<td>29.3±2.3</td>
<td>0.132</td>
</tr>
<tr>
<td><strong>Exercise training characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VO2 peak (ml/kg/min)</td>
<td>46.7±1.0</td>
<td>45.3±1.0</td>
<td>0.336</td>
<td>45.7±0.8</td>
<td>47.3±1.1</td>
<td>0.312</td>
</tr>
<tr>
<td>Ex EE (kcal/d)</td>
<td>434.4±30.4</td>
<td>382.6±30.6</td>
<td>0.233</td>
<td>391.6±24.7</td>
<td>470.5±43.0</td>
<td>0.130</td>
</tr>
<tr>
<td>Ex volume (min/wk)</td>
<td>319.3±31.5</td>
<td>361.4±40.4</td>
<td>0.407</td>
<td>327.1±27.7</td>
<td>376.1±57.8</td>
<td>0.419</td>
</tr>
<tr>
<td>Ex frequency (session/wk)</td>
<td>4.4±0.3</td>
<td>4.4±0.3</td>
<td>0.974</td>
<td>4.5±0.2</td>
<td>4.2±0.6</td>
<td>0.695</td>
</tr>
<tr>
<td>Ex intensity (kcal/min)</td>
<td>0.8±0.1</td>
<td>0.7±0.0</td>
<td>0.134</td>
<td>0.8±0.0</td>
<td>0.9±0.1</td>
<td>0.354</td>
</tr>
<tr>
<td><strong>Energy Status</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EB (kcal/d)</td>
<td>-936.1±55.6</td>
<td>-94.5±43.3</td>
<td>&lt;0.001†</td>
<td>-537.8±68.0</td>
<td>-498.3±93.3</td>
<td>0.775</td>
</tr>
<tr>
<td>EA (kcal/kg LBM)</td>
<td>25.6±1.4</td>
<td>44.2±1.0</td>
<td>&lt;0.001†</td>
<td>34.4±1.5</td>
<td>35.5±2.4</td>
<td>0.714</td>
</tr>
<tr>
<td>REE (kcal/kg LBM)</td>
<td>30.8±0.4</td>
<td>29.4±0.4</td>
<td>0.017†</td>
<td>29.6±0.3</td>
<td>32.0±0.5</td>
<td>0.001*</td>
</tr>
<tr>
<td>REE ratio</td>
<td>0.87±0.01</td>
<td>0.90±0.01</td>
<td>0.150</td>
<td>0.87±0.01</td>
<td>0.96±0.02</td>
<td>0.001*</td>
</tr>
<tr>
<td>T3 (ng/dL)</td>
<td>89.8±3.3</td>
<td>88.0±3.5</td>
<td>0.700</td>
<td>87.8±2.9</td>
<td>92.7±3.2</td>
<td>0.263</td>
</tr>
<tr>
<td>Ghrelin (pg/mL)</td>
<td>1292.7±51.3</td>
<td>1457.8±71.7</td>
<td>0.065</td>
<td>1383.5±53.5</td>
<td>1347.3±77.4</td>
<td>0.737</td>
</tr>
</tbody>
</table>

Values are reported as mean ± SEM.

OV, ovulatory; EAMD, exercise associated menstrual disturbances

EB, energy replete (≤-500 kcal/d); ED, energy deficient (≥-500 kcal/d)

EI, dietary energy intake; Ex, exercise; EE, energy expenditure; CHO, carbohydrate

*,p<0.05 vs. OV; †,p<0.05 vs. EB
Predictors of energy status

The simple odds ratios for predictors of being energy deficient are shown in Table 5-3. EA explained the greatest variance in being energy deficient ($R^2=0.827$, $p<0.001$). The log odds of being energy deficient increased by 0.62 units (38%) for every one kcal/kg LBM decrease in EA. REE also explained a significant proportion of the variance in being energy deficient ($R^2=0.084$, $p=0.021$). The log odds of being energy deficient increased by 1.2 units (20%) for every one kcal/kg LBM increase in REE. Ghrelin concentrations, body weight, REE ratio, body fat (%), BMI, and $T_3$ concentrations were not found to be significant independent predictors of being energy deficient ($p>0.05$). The adjusted odds ratio for the model best predicting an energy deficient are shown in Table 5-4. Step wise logistic regression showed that a final model including EA (kcal/kg LBM) and REE ratio accounted for 68.8% of the variance in being energy deficient.

Table 5-3. Simple odds ratio for eight predictors of being energy deficient.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Nagelkerk R Square</th>
<th>B (SE)</th>
<th>OR</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EA (kcal/kg LBM)</td>
<td>0.827</td>
<td>-0.49 (0.11)</td>
<td>0.616</td>
<td>0.49, 0.77</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>REE (kcal/kg LBM)</td>
<td>0.084</td>
<td>0.18 (0.08)</td>
<td>1.200</td>
<td>1.03, 1.40</td>
<td>0.021</td>
</tr>
<tr>
<td>Ghrelin (ng/mL)</td>
<td>0.052</td>
<td>-0.00 (0.00)</td>
<td>1.000</td>
<td>1.00, 1.00</td>
<td>0.069</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>0.034</td>
<td>0.05 (0.04)</td>
<td>1.055</td>
<td>0.98, 1.13</td>
<td>0.137</td>
</tr>
<tr>
<td>REE ratio</td>
<td>0.031</td>
<td>0.03 (0.02)</td>
<td>1.030</td>
<td>0.99, 1.08</td>
<td>0.162</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>0.014</td>
<td>0.04 (0.05)</td>
<td>1.045</td>
<td>0.96, 1.14</td>
<td>0.326</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>0.005</td>
<td>0.06 (0.11)</td>
<td>1.066</td>
<td>0.86, 1.32</td>
<td>0.557</td>
</tr>
<tr>
<td>$T_3$ (ng/dL)</td>
<td>0.002</td>
<td>0.00 (0.01)</td>
<td>1.004</td>
<td>0.99, 1.02</td>
<td>0.696</td>
</tr>
</tbody>
</table>

B, beta; SE, standard error; OR, odds ratio; CI, confidence interval

REE, resting energy expenditure; BMI, body mass index; EA, energy availability
Table 5-4. Adjusted odds ratio for predictors of being energy deficient.

<table>
<thead>
<tr>
<th>Variable</th>
<th>B (SE)</th>
<th>OR</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Included</td>
<td>7.23 (6.39)</td>
<td>0.471</td>
<td>0.30, 0.75</td>
<td>0.002</td>
</tr>
<tr>
<td>EA (kcal/kg LBM)</td>
<td>-0.75 (0.24)</td>
<td>1.242</td>
<td>1.04, 1.48</td>
<td>0.016</td>
</tr>
</tbody>
</table>

R² = 0.688 (Cox & Snell), 0.917 (Nagelkerk)

B, beta; SE, standard error; OR, odds ratio; CI, confidence interval

REE, resting energy expenditure

Predictors of menstrual status

The odds ratio for predictors of having a menstrual disturbance are shown in Table 5-5. REE ratio explained the greatest variance in having a menstrual disturbance (R²=0.304, p=0.002). The log odds of having a menstrual disturbance increased by 0.88 units (12%) for every one point decrease in REE ratio. REE also explained a significant proportion of the variance in having a menstrual disturbance (R²=0.182, p=0.002). The log odds of having a menstrual disturbance increased by 0.72 units (28%) for every one kcal/kg LBM decrease in REE. TT₃ concentrations, BMI, EA, ghrelin concentrations, body weight, energy deficit, and body fat (%) were not found to be significant independent predictors having a menstrual disturbance (p>0.05). The adjusted odds ratio for the model best predicting a menstrual disturbance are shown in Table 5-6. Step wise logistic regression showed that a final model including REE ratio accounted for 18.5% of the variance in having a menstrual disturbance (p=0.001).
Table 5-5. Simple odds ratio for nine predictors of having a menstrual disturbance.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Nagelkerk R Square</th>
<th>B (SE)</th>
<th>OR 95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>REE ratio</td>
<td>0.304</td>
<td>-0.13 (0.04)</td>
<td>0.876 0.82, 0.94</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>REE (kcal/kg LBM)</td>
<td>0.182</td>
<td>-0.33 (0.11)</td>
<td>0.719 0.58, 0.90</td>
<td>0.002</td>
</tr>
<tr>
<td>T₃ (ng/dL)</td>
<td>0.012</td>
<td>-0.01 (0.01)</td>
<td>1.000 1.00, 1.01</td>
<td>0.990</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>0.005</td>
<td>-0.07 (0.13)</td>
<td>0.934 0.72, 1.21</td>
<td>0.603</td>
</tr>
<tr>
<td>EA (kcal/kg LBM)</td>
<td>0.002</td>
<td>-0.01 (0.02)</td>
<td>0.992 0.95, 1.00</td>
<td>0.711</td>
</tr>
<tr>
<td>Ghrelin (ng/mL)</td>
<td>0.002</td>
<td>0.00 (0.00)</td>
<td>1.000 1.00, 1.00</td>
<td>0.733</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>0.001</td>
<td>0.01 (0.04)</td>
<td>1.009 0.93, 1.10</td>
<td>0.828</td>
</tr>
<tr>
<td>Energy deficit (kcal/d)</td>
<td>0.001</td>
<td>0.00 (0.00)</td>
<td>1.000 1.00, 1.00</td>
<td>0.772</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>0.000</td>
<td>0.01 (0.05)</td>
<td>1.001 0.91, 1.11</td>
<td>0.889</td>
</tr>
</tbody>
</table>

B, beta; SE, standard error; OR, odds ratio; CI, confidence interval

REE, resting energy expenditure; BMI, body mass index; EA, energy availability

Table 5-6. Adjusted odds ratio for predictors of having a menstrual disturbance.

<table>
<thead>
<tr>
<th>Variable</th>
<th>B (SE)</th>
<th>OR 95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>13.3 (3.54)</td>
<td>13.3 10.9, 16.4</td>
<td>0.001</td>
</tr>
<tr>
<td>REE ratio</td>
<td>-0.13 (0.04)</td>
<td>0.879 0.82, 0.95</td>
<td>0.001</td>
</tr>
</tbody>
</table>

R² = 0.185 (Cox & Snell), 0.296 (Nagelkerk)

B, beta; SE, standard error; OR, odds ratio; CI, confidence interval

REE, resting energy expenditure

Discussion

An important feature of this study is that the data analyzed likely represent perhaps the largest dataset available in exercising women to date where menstrual disturbances have been confirmed with measurements of estrogen, progesterone, and luteinizing hormone metabolites from daily urine samples. This was the first study to examine which of several indices of energy status best predicts the presence of an energy
deficit (ED) and menstrual disturbance in a large population of trained exercising premenopausal women. A unique feature of this study is the range of laboratory and field measures such as body weight, BMI, body composition, EA, REE, REE ratio, TT₃ concentrations, and ghrelin concentrations used to assess energy status. In addition, we collected and performed daily measures of urinary metabolites, E1G and PdG, in all of our participants for 1-3 menstrual cycles to carefully assess menstrual status.

Of these several predictors used to assess energy status, we found EA to be the most strongly associated with an energy deficit. Our results indicate that our participants were (38%) more likely to be energy deficient for every one kcal/kg LBM decrease in EA. Thus, it is possible that measuring EA in free living exercising women might serve as a useful proxy indicator of EB, which is more difficult to assess. Other studies in exercising women have documented a significant relationship between EA and EB (115, 144, 170). During short term studies manipulating EI and EEE in previously sedentary exercising women, Loucks et al. (144) showed that lower EA values were associated with similar reductions in EB. For example, an EA of 45, 30, 20, and 10 kcal/kg LBM corresponded to an energy deficit of -1, -620, -1090, and -1540 kcal/d, respectively. These studies were performed under controlled laboratory conditions though, where EI was set using a clinical dietary product (Ensure, Ross Laboratories, Columbus, OH) and EEE was monitored using indirect calorimetry. A similar relationship was observed in free living female runners (170). For example, by using the reported EI, EEE, and LBM of female runners from a published study (233), we estimated an EA value of 58.8 kcal/kg LBM for runners exhibiting an EB of +310 kcal/d and an EA value of 40.0 kcal/kg LBM for runners exhibiting an ED of -359 kcal/d. In another investigation examining the impact of
improved energy status on menstrual function in amenorrheic female athletes, using the reported EI, EEE, and FFM an estimated EA of 36.6, 31.2, 31.1, and 23.3 kcal/kg FFM can be derived for these athletes for whom authors reported an ED of -133, -754, -821, and -974 kcal/d (115). The above mentioned studies illustrate the positive relationship between EB and EA such that a decrease in EB occurs with a concomitant decrease in EA.

The physiological underpinnings of calculations of EB and EA must also be explored to understand their significant association. We operationally defined EB as EI minus the components of 24 hour energy expenditure (TDEE) which included REE, NEAT, EEE, and the thermic effect of food. Resting energy expenditure accounts for approximately 60 to 70% of TDEE, the thermic effect of food accounts for approximately 10% of TDEE, and physical activity energy expenditure which encompasses both NEAT and EEE accounts for approximately 20 to 35% of TDEE (130). Similar to Loucks et al. (144), we defined EA as EI minus EEE relative to kilograms of LBM. Since both EI and EEE are components of our EB and EA calculation, it is not surprising that EA would predict an ED. However, the calculation of EA does not account for REE, NEAT, or the thermic effect of food which together can represent approximately 80% of TDEE (130). REE has been shown to be positively associated with fat free mass in both males and females (195). Although the calculation of EA does not necessitate the measurement of REE, by expressing EA relative to LBM, it does take into account the energy associated with resting metabolic rate. We used a constant value for NEAT for our exercising women and so we cannot assess the impact of NEAT on our calculation of EB and consequently its relationship with EA. However, studies in exercising women have
shown similar NEAT values among eumenorrheic and amenorrheic runners (170, 261). Therefore, aside from TEF which represents a small percentage (10%) of TDEE (128), EA is a significant predictor of an ED in our study and it could be concluded that it may represent a useful tool in the field to estimate EB without having to measure all the components of EB which is not feasible or practical for most exercising women.

Alternatively, it must be acknowledged that the since an energy deficit is known to be related to disruptions in the menstrual cycle (62, 144, 204, 233), the strong correlation between EA and energy deficiency that we observed in this study, in the face of a non-significant association between EA and menstrual status is paradoxical. Future studies might employ a more accurate measure of energy balance, e.g., doubly labeled water in weight stable individuals, and one that does not include the same variables used in the calculation of EA with which to assess the association between energy balance and EA.

Although EA was not a significant predictor, we did find that the REE ratio best predicted having a menstrual disturbance in our large population of exercising women. In fact, participants were 12% more likely to be energy deficient for every one point decrease in the REE ratio. We compared laboratory assessed REE with a predicted REE (pREE) using the Harris-Benedict equation to estimate how much each individual’s measured REE deviated from the pREE. Most studies in women with anorexia nervosa used the Harris-Benedict equation to predict REE (114, 156, 192). During periods of low body weight and prior to refeeding, a reduced ratio of measured REE to Harris-Benedict predicted REE of 0.60 to 0.80 is often reported (114, 156, 192). De Souza and colleagues (66) reported a REE ratio less than 0.90 in estrogen and energy deficient exercising
premenopausal women which is similar to the above mentioned findings in anorexic women. Moreover, Gibbs et al. (84) showed that women with high a drive for thinness, a marker of energy deficiency, exhibited a lower measured to predicted REE ratio and greater prevalence of severe menstrual disturbances. Taken together, these studies confirm the association between menstrual disturbances and low REE:pREE ratio and support our finding of an increased odds of having a menstrual disturbance the lower estimated REE:pREE ratio.

In the current study, other markers of energy status such as body weight, body composition, TT\textsubscript{3} and ghrelin concentrations did not predict the presence of an energy deficit or menstrual disturbance. While changes in body weight are generally concomitant with alterations in reproductive function resulting from changes in EI and or energy expenditure, body weight is not always an accurate reflection of changes in EB (257, 259). Moreover, several studies have also shown that percent body fat does differ greatly among exercising women with varying menstrual status (59, 140). With regards to metabolic hormones, single measurements of TT\textsubscript{3} in energy deficient participants (10 kcal/kg LBM) have been shown to fall within the range of energy balanced participants (45 kcal/kg LBM) in well controlled laboratory experiments(133). Loucks and colleagues (135) suggested that single measurements of metabolic hormones do not reliably identify energy deficient individuals as the normal ranges across the population are wide compared to the effects of energy deficiency on them. Higher ghrelin concentrations have been reported in several studies of exercising women with amenorrhea (58-59) and adolescent athletes with anorexia nervosa (1, 38). However, ghrelin concentrations were not shown to be different among exercising women with less severe menstrual
disturbances such as luteal phase defects and anovulatory menstrual cycles or sedentary women suggesting that perhaps ghrelin is only able to discriminate the most severe energy and menstrual disturbances (58-59). The above mentioned limitations therefore provide a possible rationale as to why body composition and metabolic hormones did not best predict an energy deficit or menstrual disturbance in the current study.

The current study shows that EA and REE:pREE best predict the presence of an ED and menstrual disturbance, respectively. EA can be estimated using conventional methods such as diet logs, heart rate monitors, and exercise logs which were the methods used to compute EA in the current study. Day to day variability in EI has been reported to range from approximately 1 to 33% in females and so the accuracy of dietary intake data is a major concern (181). Heart rate monitoring and exercise logs which use the Ainsworth et al. (5) compendium to estimate the energy cost of movements have been shown to estimate energy expenditure within -4.0 to 11.4% and 7.9%, respectively, in females when compared to doubly labeled water (DLW) (47, 250). Although these methods for estimating EA are practical and feasible for most exercising women their degree of accuracy may lead to inaccuracies in prediction. REE is commonly measured under controlled laboratory conditions and has been shown to estimate energy expenditure within 15.34±2.52% when compared to DLW (212). However, a number of portable indirect calorimeters have recently been developed which provides the advantage of measuring REE in a field setting. Although data regarding the reliability and validity of these portable indirect calorimeters in measuring energy expenditure is lacking, recent studies have shown that these devices measure volumes of oxygen (VO\textsubscript{2}) and carbon dioxide (VCO\textsubscript{2}) with good agreement (VO\textsubscript{2}; r\textsuperscript{2}=0.99, VCO\textsubscript{2}; r\textsuperscript{2}=0.99) when
compared to laboratory metabolic carts (122, 147). Values of oxygen and carbon dioxide are used in the computation of energy expenditure (251). If future studies confirm the predictive roles of EA and the REE ratio for energy and menstrual status, both variables may be estimated in the field with relative ease by trained individuals.

Limitations of this study include the fact that EI used to estimate EB and EA was collected using self-reported 3-day diet logs. Several studies have shown inaccuracy when measuring energy intakes using self-reported food records, particularly related to under reporting (203). However, attempts were made to improve accuracy as much as possible. All of the participants were trained to accurately record food intake, provided with a foods amount packet which contained diagrams illustrating container sizes, cuts of meat, and various circles and squares to estimate portion sizes, and provided with a completed diet record as a reference. Another limitation is that a mean NEAT value from a reference population of exercising women was used to calculate EB. If changes in NEAT occur in a state of a positive or negative EB and thus impact the calculation of EB, we were unable to assess this effect. However, the mean NEAT value used for our calculation of EB was obtained from exercising women of similar age, BMI, and identical geographic location who also participated in a study on women’s health.

**Conclusion**

We observed that among several indices of energy status, EA and the REE ratio best predict an ED and menstrual disturbance, respectively, in a large population of exercising women. Other common measures used in the laboratory and or field setting such as T3 and ghrelin concentrations, body composition, and anthropometrics were not
found to best predict an ED or menstrual disturbance. Although EA can be measured using conventional techniques available to most exercising women such as diet logs, exercise logs, and heart rate monitoring that accuracy of these measures is a concern, and more work needs to be done to assess the association between EA and another gold standard measure of energy balance. Although the measurement of REE is typically assessed under controlled laboratory conditions, a number of companies specializing in cardiopulmonary measurements have developed portable devices to measure metabolic status in a field setting. Thus, measurements of REE ratio can be made with relative ease. If future studies continue to confirm the predictive usefulness of EA and the REE ratio, strategies to educate clinicians, registered dieticians, mental health practitioners, coaches, athletic trainers, and exercise physiologists on how to make use of these tools will be important in helping to identify exercising women at risk for the Female Athlete Triad.
Chapter 7
Discussion

The overall goal of this dissertation was to help increase our understanding of EA, defined as the difference between dietary energy intake and exercise energy expenditure normalized to kilograms of lean body mass, as a tool for assessing energy and menstrual status in a field setting and consequently its usefulness in helping to identify exercising women at risk for the Female Athlete Triad. In Study 2, we observed that EA did not discriminate menstrual status in a large sample of exercising women when using readily available conventional methods including self-reported diet logs, exercise logs, and heart rate monitoring to assess EA. Our finding agrees with other reports in the literature on much smaller numbers of subjects of which most (121, 170, 204) have observed that EA values do not differ among exercising women of varying menstrual status. Although no differences in EA were observed among our exercising women of varying menstrual status, we did observe lower TT$_3$ concentrations, REE, and ratio of REE/pREE in exercising women with severe menstrual disturbances indicating that our participants likely were exhibiting adaptations to chronic energy deficiency. It is possible that the measurement error associated with estimating EI and EEE may have contributed to the lack of a significant association between EA and menstrual status. On the other hand, the possibility that measures of other components of 24 hr energy expenditure, such as NEAT, or REE might be more tightly correlated to menstrual status should be considered. For example, if amenorrheic exercising women experienced decreases in the
energy expended outside of workouts, throughout the day i.e., NEAT, when compared to women with ovulatory cycles, then this adaptation to conserve energy may be associated with the suppression of reproductive function, but it would not be captured if one only examines EA. Likewise, several studies have demonstrated the association between the suppression of REE relative to lean body mass and amenorrhea (58) but again, EA would not capture this adaptation because REE is not included in the derivation of EA.

Although we found that EA was highly predictive of an energy deficit, our gold standard measure of energy deficiency was derived using several components of the EA index, i.e., energy intake and exercise energy expenditure and thus our gold standard measure is both very similar to our calculation of EA and wrought with the same inaccuracies.

Since an energy deficit is known to be related to disruptions in the menstrual cycle (62, 144, 204, 233), the strong correlation between EA and energy deficiency that we observed in Study 4, in the face of a non-significant association between EA and menstrual status we observed in Study 2, is paradoxical. However, since both EI and exercise energy expenditure are components of our energy deficiency calculation and our EA calculation, it is not surprising that EA would predict an energy deficiency. Moreover, EA is expressed relative to kilograms of lean body mass. Although the calculation of EA does not necessitate the measurement of REE which accounts for a large portion of 24 hr energy expenditure, LBM and REE have been shown to be highly positively correlated (195) thus making the calculation of EA and our calculation of energy balance similar and thus predictive of one another. Future studies might employ a more accurate measure of energy balance, e.g., doubly labeled water in weight stable individuals, with which to assess the association between energy balance and EA.
In order to more thoroughly assess the role of EA in reproductive status (Study 3), we also examined if trained women with menstrual disturbances who displayed reproductive recovery, whether defined as the resumption of menstrual bleeding or the resumption of menstrual bleeding preceded by ovulation, experienced a greater increase in EA when compared to trained premenopausal women with EAMD who did not display reproductive recovery. In addition, we examined if the proportion of women with EAMD who displayed reproductive recovery with an EA $\geq 30$ kcal/kg LBM was different when compared to the women with EAMD that did not display reproductive recovery. Similarly to the findings of Study 2, we did not observe a role for EA as a variable significantly associated with longitudinal changes in menstrual status, and the proportion of exercising women exhibiting an EA above the 30 kcal/kg LBM threshold in women who recovered reproductive function was not different when compared to women who did not recover reproductive function. It is possible that subtle changes in energy status were not captured with the particular variables used in this study and that perhaps other more objective laboratory measures that do not rely on self report such as leptin, ghrelin, PYY, ore measures of REE will help us to better understand the role of changes in energy balance in the recovery of menstrual function. It is also possible that the lack of observed changes in EA and other indices of energy status are largely a result of the small sample size, as some trends toward significance were observed. Future studies with a larger sample size and more accurate measures of EA are needed to confirm its role alongside other energy related variables in the recovery of reproductive function.

Another important finding of these studies is that among several indices of energy status including body composition, EA, REE, and metabolic hormones, REE:pREE was
found to best predict a menstrual disturbance (Study 4). A number of studies have used the above mentioned indices of energy status to corroborate an energy deficiency, adaptations to conserve energy, and the risk of menstrual disturbances (58, 84, 144). However, no studies to date have examined which is the best predictor or how well these measures compare to one another. This gap in the literature was the underpinning for addressing this important question. It was not surprising that EA did not best predict a menstrual disturbance as we observed in Study 2 that EA did not discriminate normal ovarian function from either subtle (luteal phase defects or anovulation) or severe (oligomenorrhea or amenorrhea) menstrual disturbances in a large population of exercising women. Our finding is similar to other studies that have documented lower REE:pREE in exercising women with menstrual disturbances (58, 66, 196). This finding is also similar to the results of Study 2 such that we observed a lower REE:pREE in exercising women with severe menstrual disturbances. The measurement of REE is typically assessed under controlled laboratory conditions; however, a number of companies specializing in cardiopulmonary measurements have developed portable devices to measure metabolic status in a field setting. The data regarding the reliability and validity of these portable indirect calorimeters in measuring energy expenditure is lacking at the present time (122, 147). Thus, measurements and REE:pREE can be made with relative ease. Educating clinicians, registered dieticians, mental health practitioners, coaches, athletic trainers, and exercise physiologists about the best methods to predict an energy deficit and the presence of menstrual disturbances (especially subtle menstrual disturbances) is important in helping to identify exercising women at risk for the Female Athlete Triad.
Lastly, we demonstrated that although the mean EA was not low (< 30 kcal/kg FFM) at any point in the season when all subjects are considered, a concerning percentage of the soccer players exhibited low EA at some point in the season (Study 1). This finding is not surprising as female athletes have been shown to consume low EI (40) relative to high EEE (213). In addition, an unexpected high prevalence of eating disorders (28%) among female athletes participating in ball-game sports has been reported (224) and disordered eating attitudes have been shown to predispose female athletes to consume lower EI (84, 196). In line with this latter finding, we observed negative eating attitudes, in particular high body dissatisfaction, in our Division I female soccer players with low compared to higher EA. Changes in EA appeared to be reversible and seasonal as few Division I female soccer players showed low EA during the post season. We observed lower EI at lunch and dinner during the pre season as well as breakfast and lunch during the mid season in female soccer players who presented with low EA. This result was also not surprising as Tomten and Hostmark (233) reported lower EI at dinner in energy deficient runners with irregular menstrual function. This finding is of significant translational value as nutritional strategies that focus on monitoring EI at meals, particularly lunch, in addition to eating attitudes may help athletes achieve the nutritional guidelines put forth by the American College of Sports Medicine for nutrition and athletic performance. The results of Study 1 suggest that although the majority of soccer players were not energy deficient a small percentage of soccer players were. Whether the women who displayed low EA are at risk for the Triad is debateable however, since the results of Studies 2 and 3 show that low EA was not found to discriminate menstrual status in a large cross sectional sample of exercising women or to
increase significantly in those who recovered reproductive function. As well, the physiological relevance of low EA in these athletes is not clear, as this was not corroborated by suppressed TT$_3$ concentrations.

**Conclusion**

These studies include analyses from the largest dataset to date to assess the usefulness of EA as tool for assessing energy and menstrual status in a field setting and consequently its usefulness in helping to identify exercising women at risk for the Female Athlete Triad. Consequently, these findings add to the small but growing number of reports from studies attempting to determine the association between EA as an index of energy status and menstrual function in exercising women in a field setting (196, 204). Our data suggest that EA may not be a useful tool in assessing menstrual exercising women. Although we showed that EA best predicts an energy deficit, more research using a more accurate gold standard measure to represent energy balance is likely necessary, since EA showed no association with menstrual disturbances. Additional exploration of the lack of association between EA and menstrual function deserves further attention as other factors such as psychological stress may be contributed to the EAMD we characterized. Future studies are needed to assess the accuracy of the calculation of EA and other indices of energy deficiency and how changes in one might affect the other. As well, the translational potential for using the index of REE:pREE to discriminate menstrual status should be examined.
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