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THE EFFECT OF MENSTRUAL CYCLE PHASE ON APPETITE-REGULATING HORMONES

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THE EFFECT OF MENSTRUAL CYCLE PHASE ON APPETITE-REGULATING HORMONES

by

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Abstract

Background: Obesity rates are higher among females than males potentially due to changing food intake across the menstrual cycle. Food intake is partially regulated by circulating appetite-regulating hormones that can be influenced by the menstrual hormones estradiol and progesterone. **Purpose:** Examine whether changes in female sex hormones across the menstrual cycle are related to appetite-regulating hormones. **Methods:** 8 healthy young females were tested during the follicular, ovulatory, and luteal phases; phases were confirmed by fasting plasma hormone concentrations. Blood samples were taken after an overnight fast, as well as 30, 60, and 90 min post-prandially to test the concentrations of acylated ghrelin and active GLP-1. Perceived hunger, fullness, satisfaction, and prospective food intake were collected immediately prior to blood samples. Resting metabolic rate and substrate oxidation were tested through indirect calorimetry. **Results:** Menstrual cycle phase had no effect on acylated ghrelin (P=0.959) or active GLP-1 (P=0.650). Post-prandial fullness was greater during the OP compared to FP (P=0.02) and approached significance with the LP (P=0.06) but there was no difference in other perceptions of appetite. There was no effect of menstrual cycle phase on resting metabolic rate (P=0.961), carbohydrate oxidation (P=0.603), or fat oxidation rate (P=0.485). **Conclusion:** Acylated ghrelin and active GLP-1 are unlikely to explain the previously documented changes in energy intake across the menstrual cycle though perceived fullness can be elevated during the luteal phase. Taken together, this suggests that sensitivity to appetite-regulating hormones might fluctuate across the menstrual cycle.

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List of Acronyms

AG: acylated ghrelin

AgRP: agouti-related peptide

ARC: arcuate nucleus

BMI: body mass index

CART: cocaine and amphetamine related transcript

E_2: estradiol

FP: follicular phase

FSH: follicle-stimulating hormone

GLP-1: glucagon-like peptide-1

kcal: kilocalorie

LH: luteinizing hormone

LP: luteal phase

mRNA: messenger ribonucleic acid

NPY: neuropeptide Y

OP: ovulatory phase

P_4: progesterone

POMC: pro-opiomelanocortin

RER: respiratory exchange ratio

Chapter 1 - Literature Review

Background:

Obesity has become a worldwide epidemic afflicting ~1.6 billion people worldwide (1). A majority of Canadians are currently overweight or obese (2) with an economic burden of $4.3 billion in 2004, ~2.5% of total health care costs (3). Obesity is generally caused by a sustained increase in energy intake (i.e. over eating) and decreased energy expenditure (i.e. increasingly sedentary behaviour). Together these produce a chronic positive energy balance that inevitably leads to increased storage of energy, primarily as fat. At the same time, rates of obesity (body mass index >30) and morbid obesity (body mass index >40) are higher among females than males (4,5) and overweight females are more likely to become obese compared to overweight males (6). Unfortunately, despite ~52% of Canadian females currently trying to lose weight (7), exercise interventions are often far less effective at improving body composition in females than males (8-11). The difficulty females experience with weight management might be partly explained by fluctuations in female sex hormones, namely estradiol and progesterone, associated with the menstrual cycle as these hormones are known to influence energy intake and energy expenditure (12-16). Unfortunately, females remain largely under-researched in physiology (17) making it imperative to conduct studies on female-specific physiology to understand the aforementioned problems that females experience with weight management.

Appetite Regulation:

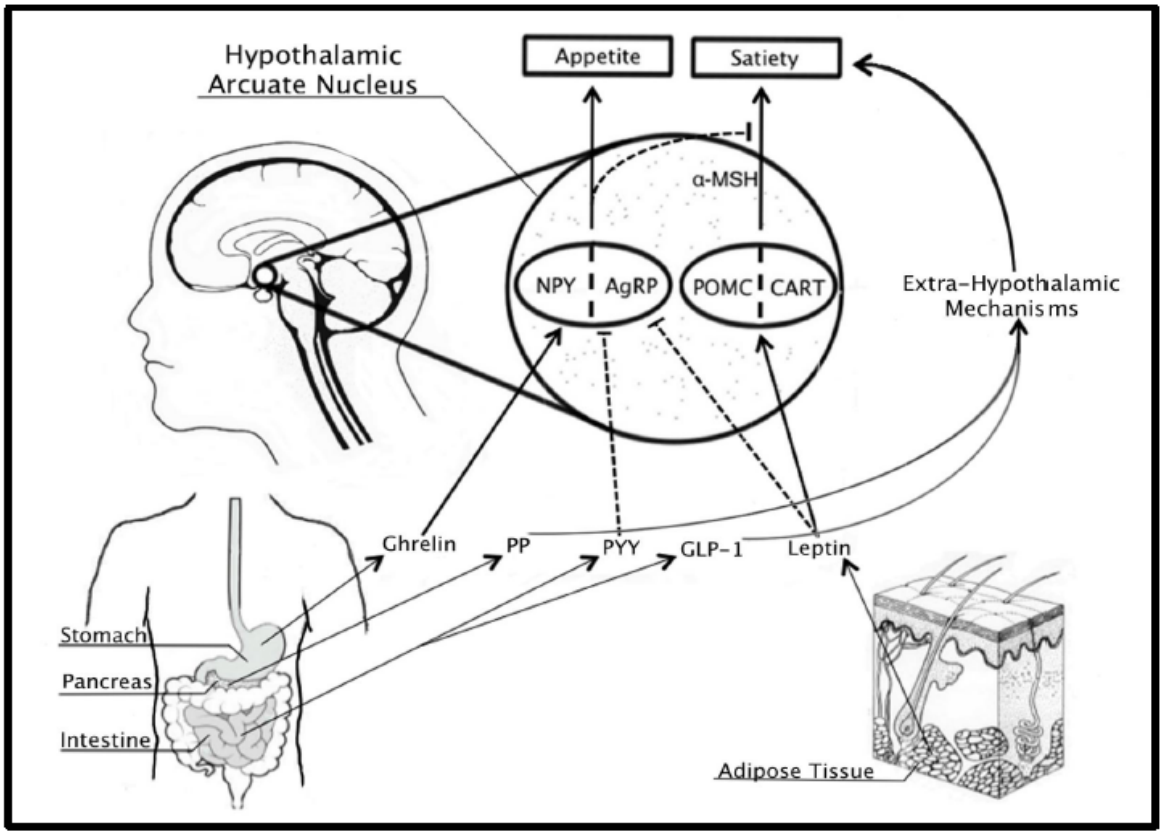
Overview. The control of energy intake is a complex system incorporating psychological, social, environmental, behavioural, and physiological components (18). The complexity of appetite regulation makes it exceedingly difficult to determine how any single factor will contribute to actual energy intake. Moreover, the difficulty controlling for environmental and social factors

makes understanding the more readily modifiable factors that influence eating behaviour, such as appetite-regulating hormones, important for developing our understanding of appetite regulation. Physiologically, circulating gut-derived endocrine signals acutely respond to feeding and also reflect total adiposity and energy balance to regulate energy intake (19). These circulating hormones are integrated in the arcuate nucleus (ARC) of the hypothalamus to mediate hunger, satiety, and energy intake (20,21). Currently the only known orexigenic (appetite-stimulating) gut-derived hormone is ghrelin, secreted from the stomach, that induces hunger by stimulating the NPY and AgRP neurons in the ARC (22). Whereas, there are numerous anorexigenic (satiety-inducing) hormones, including glucagon-like peptide-1 (GLP-1) that creates satiating effects through delaying gastric emptying and incretin effects (20) (Figure 1).

Ghrelin. Ghrelin is a 28-amino acid peptide hormone synthesized predominantly by specialized cells in the stomach (22). Recently it has become possible to accurately measure acylated ghrelin (AG), the active form of ghrelin. Acylation of ghrelin involves the addition of an octanoyl group by the enzyme ghrelin O-acyl transferase (23) and is rapidly degraded if not inhibited (22,24). Although AG represents only 10-20% of total circulating ghrelin it is believed to be more important in regards to appetite stimulation (25).

In animal models, exogenously administered ghrelin increases energy intake (26). Similarly, elevated concentrations of ghrelin stimulates energy intake in humans (26). Moreover, circulating ghrelin concentrations increase while fasting and rapidly decrease after eating (26-28), peaks in ghrelin precede increased perceptions of hunger by only a short time (29), are strongly associated with perceptions of hunger (30). Taken together, these data

Figure 1: Schematic of gut-derived appetite-regulating hormones and the mechanisms through which they control energy intake. Ghrelin stimulates hunger by interacting with NPY/AgRP neurons in the arcuate nucleus. GLP-1 functions through non-hypothalamic mechanisms, including incretin effects and delaying gastric emptying. Continuous lines represent stimulatory effects while dashed lines represent inhibitory effects.



implicate ghrelin in the stimulation of hunger and energy intake. Concentrations of AG are suppressed within min of food consumption and begin to rise around 1.5-2 h post-prandially (30), although this can be modulated by various factors, including macronutrient composition (31) or an individual's body composition (32).

Glucagon-like peptide (GLP-1). GLP-1 is an anorexigenic gut-derived peptide secreted primarily from the small intestine (33). Bioactive GLP-1 is generated from two circulating forms, GLP-$1_{7\text{-}37}$ and GLP-$1_{7\text{-}36}$ (34). Both forms of GLP-1 are equally potent (34) but in human plasma GLP-$1_{7\text{-}36}$ represents the majority of active circulating active GLP-1 (35). Following

release, GLP-1 is rapidly degraded by dipeptidyl peptidase-4 (DPP-IV) to its inactive form GLP-$1_{9\text{-}36}$ (34).

In animals, the absence of GLP-1 produces bodyweight gain due to increased energy intake compared to GLP-1 replete animals (36). In humans, increased concentrations of GLP-1 decrease hunger (37) and reduce energy intake (38). These data reflect GLP-1's anorexigenic effects. GLP-1 concentrations begin to increase within min of food ingestion and remain elevated for ~1 h (33). Similarly to ghrelin, secretion of GLP-1 can be influenced by macronutrient composition (39) or an individual's body composition (40). The mechanisms by which GLP-1 induces satiety likely include stimulating insulin secretion, inhibiting the release of glucagon, and delaying gastric emptying (41).

The Menstrual Cycle:

Basic Physiology. The menstrual cycle is regulated by the interaction of pituitary hormones, including gonadotropin-releasing hormone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and the ovarian hormones progesterone and estrogen (Figure 2) (42). There are various forms of estrogen but estradiol is the most biologically active form in young healthy women (16,17). The female menstrual cycle can be divided into discrete phases based on its hormonal profile. For instance, the follicular phase (FP) beings with menses, ends at ovulation, and is characterized by relatively low concentrations of estradiol and progesterone (43). Near the end of the FP there is a surge of estradiol that triggers the secretion of LH and FSH to induce ovulation. Once ovulation (i.e. release of the oocyte/egg) has occurred, the resulting corpus luteum within the ovary begins to secrete large amounts of progesterone. Consequently, the luteal phase (LP) is characterized by a progesterone-dominated state, although estradiol is also

elevated relative to the FP (44). The LP lasts from ovulation until menses of the next menstrual cycle (45). While previous research has demonstrated that gonadotropin-releasing hormone, LH, and FSH do not correlate with eating in female rats (46), estradiol and progesterone play an important role in energy intake.

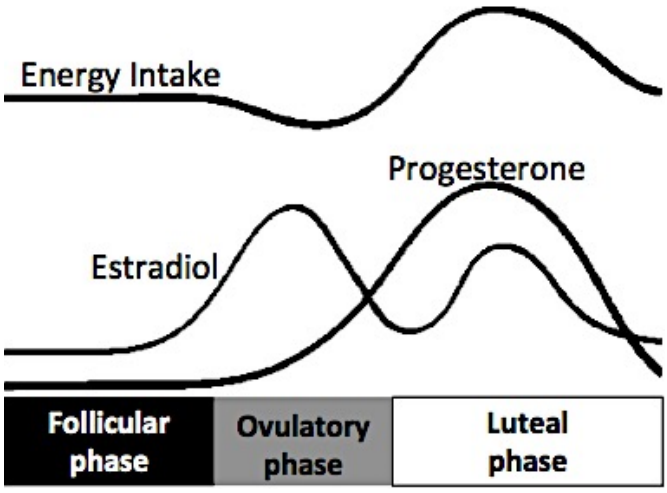
Menstrual Cycle and Energy Intake. It is well documented that energy intake fluctuates throughout the menstrual cycle and that changes coincide with fluctuations in ovarian hormones (15,47-49) (Figure 3). Daily energy intake is lowest during ovulation (13,14,46) which is attributed to the anorexigenic effects of estradiol. Following ovulation, energy intake increases by up to 90-500 kcal/day, or an increment of 3.9-34.7%, during the LP compared to the FP (15,50-53). The increased energy intake potentially reflects the anti-estrogenic and orexigenic properties of progesterone (45,54). Indeed, females may consume ~10% more during one-half of their menstrual cycle which is certainly capable of influencing energy balance (46) and eventually body weight. Importantly, these changes in energy intake are explained entirely by differences in the caloric intake at each meal, as opposed to changes in the frequency of energy intake (46,55). Therefore, it seems reasonable to speculate that distinct hormonal responses occur post-prandially during each menstrual phase. For example, AG might decrease more rapidly or profoundly post-prandially during the OP, thereby reducing perceptions of hunger and energy intake in this phase. In this light, it is particularly important for research to investigate how appetite-regulating hormones respond to feeding throughout the menstrual cycle.

Menstrual Cycle and Appetite-Regulating Hormones. In light of the aforementioned changes in energy intake across the female menstrual cycle, it is conceivable that female sex hormones are

altering hormones that regulate energy intake. Estradiol is a potent anorexigenic agent that is at least partly responsible for the decreased energy intake during ovulation (48,49). Estradiol is an inhibitory signal that links the hypothalamic–pituitary–gonadal axis to the neural control of feeding and decreases energy intake by reducing the quantity of food consumed at each meal (43). Estradiol's anorexigenic effect can be observed in ovariectomized rats who consequently express very low concentrations of estradiol, hyperphagia, and a 10-30% increase in body fat (56) and the reversal of these effects by estradiol treatment alone (57).

Estradiol may produce its anorexigenic effect through interactions with appetite-regulating gut hormones. Indeed, diminished numbers of ghrelin-producing cells, ghrelin mRNA concentrations in the stomach, and plasma ghrelin concentrations were all observed when estradiol was exogenously administered to ovariectomized rats (58). Furthermore, total ghrelin no longer stimulated energy intake in ovariectomized rats who received estradiol infusions, implying that the orexigenic potency of ghrelin is diminished by estradiol (16). Finally, human females with hypothalamic amenorrhea who consequently exhibit lower than average estradiol concentrations also express concomitantly greater (44%) total ghrelin concentrations compared to their estradiol-replete counterparts (59).

Figure 2: Fluctuations in female sex hormones and energy intake across the menstrual cycle. Adapted from (Hirschberg et al. 2012)1



As ghrelin producing cells possess estradiol receptors (58), this suggests a direct mechanism for how AG production could be influenced by estradiol, it is less clear how GLP-1

could be effected. One potential mechanism is altered gastric emptying throughout the menstrual cycle. Gastric emptying is an important regulator of GLP-1 secretion (60), but currently there are conflicting reports on whether gastric emptying is altered by female sex hormones (61-63). At the same time, there is evidence that estradiol can increase the satiating potency of GLP-1, although it is unclear how this occurs given that central GLP-1 receptors do not possess estrogen receptors (46).

Estradiol can also influence appetite and energy intake through the stimulation of estradiol receptors located in various regions of the brain, especially the hypothalamus. This is supported by an observable decrease in eating when dilute estradiol is implanted directly into the hypothalamus in ovariectomized rats (64). Furthermore, the NPY/AgRP and POMC/CART neurons within the hypothalamus possess abundant estradiol receptors so it is possible that estradiol exerts its satiety effect directly through these neurons which play important roles in the regulation of energy intake (65-67).

An important limitation of utilizing the menstrual cycle to test the effects of sex hormones is that progesterone cannot be isolated since there is no time when progesterone alone is elevated (17). For this reason, the effects of progesterone are less understood than estradiol. However, while progesterone alone does not seem to significantly alter energy intake in ovariectomized rats unless it is administered in supraphysiological doses (68) there is support for an orexigenic effect of progesterone in human females (69). In particular, the increased hunger accompanying pregnancy has been partially attributed to the effects of progesterone on specific neurons in the ARC (70). Also, food cravings are more frequent and intense during the LP which has been attributed to progesterone (15,69). It is possible that the orexigenic effect of progesterone is actually due to its antagonizing and suppressing estradiol's effects. Many of

estradiol's metabolic actions are antagonized by progesterone (45) so it may be that progesterone can inhibit the anorexigenic effect of estradiol. Although it is not exactly clear how this happens, when progesterone levels were artificially elevated in rhesus monkeys, estradiol levels were diminished and the estradiol surge that precedes ovulation did not occur (71). Regardless of the specific mechanism, progesterone seems able to stimulate energy intake and weight gain suggesting that it could contribute to the greater energy intake in the progesterone-dominated LP.

Menstrual Cycle and Energy Expenditure. To fully understand how the menstrual cycle could influence weight maintenance it is important to examine both components of energy balance, namely energy intake and energy expenditure. The greatest component of daily energy turnover is generally resting metabolic rate (72). Studies consistently indicate that daily energy expenditure fluctuates throughout the menstrual cycle (73-77). During the LP, energy expenditure can increase up to 279 kcal/day, or 2.5-11.5%, compared to the FP (43). Elevated energy expenditure during the LP may result, at least in part, from the hyperthermic effects of progesterone (45). Moreover, many of the changes that occur during pregnancy, a very energetically costly and progesterone-dominated state, are also observed during the LP, including a 0.3-0.5 °C increase in body temperature (45). Taken together, these data demonstrate the importance of considering both energy expenditure and energy intake when exploring how the female menstrual cycle affects weight management.

Teleological Explanation. Compared to males, females consistently demonstrate greater maintenance of energy balance (>99.5% accuracy over a year) in response to acute energy

deficits (72,78-80). This precise maintenance is probably driven by the relationship between energy balance and reproductive success in females (81). In females, even a modest energy deficit can suppress ovulatory cycles and inhibit the hypothalamic–pituitary–gonadal axis (82) while there appears to be no major disturbances in reproductive function when large energy deficits exist in males (81). Because lactation and parental thermoregulatory behaviours are extremely energetically costly in mammals (83) it seems intuitive that a hormonal milieu able to drive energy intake would exist during pregnancy. Indeed, the increased progesterone and estradiol during pregnancy are similar to that during the LP. Therefore, the LP may represent a "gestational-preparation stage" whereby females are preparing for pregnancy to occur, as it would around ovulation, the stage immediately before the LP. This would be an evolutionarily pertinent function as even a small energy deficit at the early stages of pregnancy could have detrimental effects on fetal development.

Summary:

Females experience predictable fluctuations in ovarian hormones across the menstrual cycle and these hormones can influence energy intake and energy expenditure. Past research on appetite-regulating hormones has focused on males, with the only studies on females occurring during the early FP (84-86) when hormone concentrations are lowest. Although informative, studying one phase that represents only ~50% of adult female life provides an incomplete understanding and can lead to inappropriate conclusions. Considering the interactions between sex and appetite-regulating hormones it seems reasonable that these could be at least partially responsible for the changes in energy intake across the menstrual cycle.

Therefore, the purpose of the current project was to investigate the effect of menstrual phase on appetite-regulating hormones and perceptions of appetite. We hypothesize that

compared to the OP and FP, there will be elevated concentrations of AG, perceived hunger, and low active GLP-1 during the LP, while progesterone is increased. Conversely, during OP, with its elevated estradiol concentrations, there will be lower AG but greater concentrations of active GLP-1 and a concomitantly reduced hunger compared to both the FP and LP. These results will provide important information on the mechanisms of weight management in females.

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Chapter 2

Menstrual cycle phase has no effect on acylated ghrelin and GLP-1

Abstract

Background: Obesity rates are higher among females than males potentially due to changing food intake across the menstrual cycle. Food intake is partially regulated by circulating appetite-regulating hormones that can be influenced by the menstrual hormones estradiol and progesterone. **Purpose:** Examine whether changes in female sex hormones across the menstrual cycle are related to appetite-regulating hormones. **Methods:** 8 healthy young females were tested during the follicular, ovulatory, and luteal phases; phases were confirmed by fasting plasma hormone concentrations. Blood samples were taken after an overnight fast, as well as 30, 60, and 90 min post-prandially to test the concentrations of acylated ghrelin and active GLP-1. Perceived hunger, fullness, satisfaction, and prospective food intake were collected immediately prior to blood samples. Resting metabolic rate and substrate oxidation were tested through indirect calorimetry. **Results:** Menstrual cycle phase had no effect on acylated ghrelin (P=0.959) or active GLP-1 (P=0.650). Post-prandial fullness was greater during the OP compared to FP (P=0.02) and approached significance with the LP (P=0.06) but there was no difference in other perceptions of appetite. There was no effect of menstrual cycle phase on resting metabolic rate (P=0.961), carbohydrate oxidation rate (P=0.603), or fat oxidation rate (P=0.485). **Conclusion:** Acylated ghrelin and active GLP-1 are unlikely to explain the previously documented changes in energy intake across the menstrual cycle though perceived fullness can be elevated during the luteal phase. Taken together, this suggests that sensitivity to appetite-regulating hormones might fluctuate across the menstrual cycle.

KEYWORDS: GHRELIN, GLP-1, APPETITE, MENSTRUAL CYCLE, FEMALE, RESTING METABOLIC RATE

Introduction

Despite ~50% of North American females currently trying to lose weight (1,2), overweight females are more likely to become obese compared to overweight males (3) partly explaining the greater rates of obesity (body mass index >30) and morbid obesity (body mass index >40) among females (4,5). Moreover, university is an important time as 60% of first year students gain 3.5 kg (6). In light of this, it is important we explore the regulation of energy intake by healthy young females to possibly prevent the progression towards unhealthy body weight gain. The difficulty females experience with weight management might be explained by female sex hormones, namely progesterone (P_4) and estradiol (E_2), the most biologically potent form of estrogen (7,8). These hormones fluctuate across the menstrual cycle and are known to influence energy intake and energy expenditure (7,9-12).

The female menstrual cycle can be divided into three phases based on their associated hormonal milieu. The first phase is the follicular phase (FP) that is characterized by relatively low concentrations of both E_2 and P_4 and begins with menses (day 1) and continues until ovulation (approximately day 12) (13). Immediately prior to ovulation (OP), between days 7-10, there is a surge in E_2 but no change in P_4 (13). The third phase is the luteal phase (LP) during which time both E_2 and P_4 concentrations are elevated compared to the FP but is dominated by P_4 and lasts from ovulation until the next menses. Interestingly, relative to the FP, energy intake consistently decreases around OP and increases during the LP (11,12,14,15).

Estradiol has potent anorexigenic (satiety-inducing) effects reflected by the reduced energy intake around ovulation (11,16). Moreover, there is evidence that E_2 can influence the production of ghrelin (17) as well as the potency of ghrelin (7), leptin (18), and cholecystokinin (19). Conversely, the orexigenic (appetite-inducing) effect of P_4 can be seen by the increased

energy intake during the LP when P_4 concentrations spike (12,20-22). It is not exactly clear how P_4 affects energy intake, although it does have anti-estrogenic qualities (23) and thus might suppress E_2's anorexigenic effects thereby producing an orexigenic drive. There is also evidence that menstrual cycle phase can alter the production of GLP-1 (24). Importantly, during anovulatory cycles, where P_4 does not increase in the LP, energy intake no longer increases (12) supporting that the fluctuating sex hormones are responsible for changing energy intake (21,25,26).

The physiological control of energy intake is partly mediated by circulating gut-derived hormones that either stimulate appetite (i.e. orexigenic) or induce satiety (i.e. anorexigenic) (27). Currently the only known orexigenic gut hormone is ghrelin, synthesized predominately by specialized endocrine cells in the stomach, and then activated by the addition of an octanoyl group to acylated ghrelin (AG) (28,29). Importantly, peaks in ghrelin precede increased perceptions of hunger by only a short time (30) and are strongly associated with hunger and energy intake (31). Although AG represents only 10-20% of total circulating ghrelin, it is thought to be more important in terms of appetite regulation (32,33). In opposition, various hormones such as glucagon-like peptide-1 (GLP-1) induce satiety (27). Active GLP-1 exists in two equipotent forms, GLP-1_{7-36} and GLP-1_{7-37}, although GLP-1_{7-36} represents a majority of the circulating active GLP-1 (34). GLP-1 is secreted within min of food ingestion and persists for up to one h post-prandially (35). GLP-1 can suppress appetite (36), reduce energy intake (37), and active GLP-1 is negatively associated with hunger and energy intake (31). Together, these data support that these gut-derived hormones are important in appetite regulation and energy intake. There is evidence that female sex hormones can influence AG (17,38) and GLP-1 (24)

but it is not known whether fluctuations in E_2 and P_4 across the menstrual cycle can have an effect.

To gain a more comprehensive understanding of the effect of menstrual cycle phase on energy balance it is important to examine both energy intake and energy expenditure. Indeed, it might be speculated that a change in energy expenditure could alter energy intake, or vice versa. A number of studies indicate that 24-h energy expenditure can increase by as much as 89–279 kcal/day during the LP compared to the FP (13). Accordingly, increased sleeping metabolic rate (39,40), basal metabolic rate (41), and 24-h energy expenditure (42,43) have all been reported during the LP. Thus, because energy expenditure and energy intake can fluctuate across menstrual phases it is important to simultaneously collect these data from the same group of females across a single menstrual cycle.

Changes in energy intake across the menstrual cycle are explained entirely by differences in the caloric intake at each meal, as opposed to changes in the frequency of energy intake (19,44). Therefore, it seems reasonable to speculate that distinct post-prandial hormonal responses would occur during each menstrual phase. For example, post-prandially AG might decrease more rapidly or profoundly during the OP, thereby reducing perceptions of hunger and energy intake in this phase. Therefore, the purpose of this project was to investigate fasting and post-prandial AG and active GLP-1 concentrations throughout the menstrual cycle. We hypothesized that the LP, with increased P_4, would be associated with greater concentrations of AG, low active GLP-1, and a greater perceived hunger. During OP, with high concentrations of E_2, there would be lower AG but greater concentrations of active GLP-1, and a concomitantly diminished perceived hunger. These results will provide important information on appetite-regulation in females.

Methods

Participants. Eight healthy females volunteered to participate in the current project (age: 23.5±5.0 y; BMI: 24.15±3.15 $kg \cdot m^{-2}$). Prior to study initiation, the experimental procedures and potential risks were explained fully, and all participants provided written, informed consent. The Wilfrid Laurier University Ethics Committee for Research on Human Subjects approved this study.

Participants were between the ages of 18 and 35, recreationally active (exercising ≤3 sessions/wk), healthy, non-smokers, eumenorrheic for at least 1 y (regularly occurring menstrual cycle lasting 24-35 d) based off self-reported menstrual history (23), menstruating for at least 3 y (45), and had not been pregnant in the past 3 y nor were planning to become pregnant at the time of data collection. Females were either non-hormonal contraceptive users for a minimum of 6 months or were using a tri-phasic hormonal contraceptive continuously for >1 y. Tri-phasic pills suppress endogenous hormone production thereby providing a consistent and predictable hormonal milieu while decreasing inter- and intra-variability in hormone concentrations (46). Despite having far greater biological activity (up to 100X compared to natural forms) (47), synthetic sex hormones in combined oral contraceptives produce a hormonal environment similar to naturally fluctuating females (46). Moreover, tri-phasic contraceptives have been included in past studies on appetite-regulating hormones (48,49) and various forms of oral-contraceptives do not affect food choice or energy intake (50,51). Participants provided a detailed menstrual cycle history of at least the past three cycles. Day 1 of the menstrual cycle was characterized by the onset of menses. Menstrual phase was subject-monitored by oral body temperature taken upon awakening with a sustained increase of ~0.3 ºC identifying ovulation

(23); participants reported their temperature each morning via email. All phases were subsequently confirmed by hormonal testing of fasting plasma E_2 and P_4 collected on study days.

Experimental Design. Participants had their hormonal concentrations and energy expenditure measured during all three menstrual phases. Additionally, during each phase we assessed perceptions of hunger, fullness, prospective energy intake, and food preference using visual analogue scale (VAS). Each participant underwent a familiarization session before the first experimental session followed by three experimental sessions (~3 h). During familiarization, participants became accustomed with the respiratory mask, VAS, and blood sampling procedures. Participant sessions were systematically rotated to minimize any order effects.

Participants were instructed to not engage in any strenuous physical activity for 48 h and to refrain from caffeine and eating the morning of a session. Participants kept detailed food logs 24 h before all sessions such that energy intake could be replicated before subsequent sessions. Participants arrived at the laboratory at 0800 h after an overnight fast (>10 h). Upon arrival, participants were fitted with a respiratory mask that was worn continuously for 30 min. Following gas collection, appetite was assessed using VAS. Immediately afterward, the first blood sample was collected (0845 h) to test fasting hormone concentrations. After the first blood sample participants were served a standardized breakfast (chocolate chip Clif bar, CA, USA; 29 $kJ \cdot kg^{-1}$ body weight) to be consumed within 15 min and were given 30 min for digestion. After this, participants completed three VAS assessments immediately before blood samples collected 30 (0930 h), 60 (1000 h), and 90 min post-prandially (1030 h); similar sampling times have been used in the past to determine changes in AG (32,33) and GLP-1 (52).

Gas Collection. Gas exchange was measured continuously for 30 min prior to eating. All gas exchange measurements (VO_2, VCO_2, respiratory exchange ratio) were made using an online

breath-by-breath gas collection and analysis system (Max II, AEI Technologies, Pittsburgh, PA, USA). Prior to data collection the gas analyzer was calibrated with gases of known concentrations and flow with a 3-L syringe. All measures were collected while participants were seated in a temperature-controlled room (~21 °C). The resting VO_2 and VCO_2 ($L \cdot min^{-1}$) values were averaged over the last 15 min of gas collection and used to determine the rates of fat and carbohydrate oxidation. Resting metabolic rate (RMR) was estimated using total VO_2 ($L \cdot min^{-1}$) over the final 15 min and an assumed relationship of 5 kcal per litre of O_2. The following stoichiometric equations were used with the assumption that protein oxidation was negligible (53):

$$\text{Fat oxidation (g/min)} = 1.695 \cdot VO_2\ (L \cdot min^{-1}) \cdot 1.701 \cdot VCO_2\ (L \cdot min^{-1})$$

$$\text{Carbohydrate oxidation (g/min)} = 4.210 \cdot VCO_2\ (L \cdot min^{-1}) \cdot 2.962 \cdot VO_2\ (L \cdot min^{-1})$$

Appetite Perceptions. Participants recorded perceptions of hunger, fullness, satisfaction, and prospective EI using VAS (54). Responses were recorded upon arriving at lab (fasting) and immediately before every blood draw. The VAS involved marking along a 100 mm line in response to questions such as: " How often do you eat breakfast in the morning?"; "How hungry do you feel right now?"; "How full do you feel right now?"; "How satisfied do you feel right now?"; and "How much food could you eat right now?". The assessment of these questions utilizing VAS has been validated (54) and used in past appetite research (55).

Blood Sampling. Two 3-mL venous blood samples were collected from an antecubital vein at 0845 h (#1; fasting), 0930 (#2; 30 min post-prandial), 1000 (#3; 60 min post-prandial), and 1030 h (#4; 90 min post-prandial) while participants were in the supine position. Blood samples were collected into pre-chilled vacutainer tubes (BD vacutainer K2 ethylenediaminetetraacetic acid [EDTA; lavender top]; Becton, Dickinson and Company, NJ, USA). Inhibitors were

immediately added for hormone preservation. To preserve AG, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride was added (AEBSF; 10 μL per 1 mL of whole blood) to the first vacutainer tube. To prevent inactivation of GLP-1, 10-μL of DPP-IV inhibitor and 500 KIU aprotinin per mL whole blood was added to the second vacutainer tube. Estradiol and progesterone samples were analyzed from samples containing AEBSF, similar to past reports (56-58).

All tubes were gently inverted ~10 times and centrifuged at 3000 rpm for 10 min at 4°C. The plasma supernatant was then aliquoted into microcentrifuge tubes. To preserve AG, plasma was acidified by the addition of 100 μL of 1 M HCl per mL plasma (50 μL). All samples were stored at -80°C for subsequent analysis.

Biochemical Analysis. Commercially available enzyme-linked immunosorbent assays (ELISA) were performed according to manufacturer recommendations to determine plasma concentrations of AG (Cat. # EZGRA-88K, EMD Millipore, MA, USA), active GLP-1 (Cat. # EGLP-35K, EMD Millipore, MA, USA), E_2 (SKU: DCM003, Eagle Biosciencesm, Inc., NH, USA), and P_4 (SKU: DCM025, Eagle Biosciencesm, Inc., NH, USA). The acylated ghrelin assay was specific for measuring the active (acylated) form of this peptide in human serum or plasma with no cross reactivity with inactive (des-acyl) ghrelin. The active GLP-1 assay was specific for measuring the two active forms of this peptide ($GLP\text{-}1_{7\text{-}36}$ and $GLP\text{-}1_{7\text{-}37}$) in human plasma with no cross reactivity (<3%) with other forms of GLP-1 (i.e. $GLP\text{-}1_{1\text{-}36}$, $GLP\text{-}1_{1\text{-}37}$, $GLP\text{-}1_{9\text{-}36}$, $GLP\text{-}1_{9\text{-}37}$). Estradiol assay had no cross reactivity (<2%) with other forms of estrogen and the progesterone assay did not cross react with testosterone (<1%) or other progesterones (<1%). To eliminate inter-assay variation, samples from each participant were analyzed in the same run. Samples were run in duplicate to reduce intra-assay variation. The intra-assay coefficient of variation was

8.41±5.66 for AG, 11.07±8.29 for active GLP-1, 6.01±5.82 for E_2, and 5.16±5.93 for P_4; all were below the accepted standard of detection for each kit.

Statistical analysis. Data were analyzed using SPSS software version 22.0 for Windows (SPSS, Chicago, IL). One-way repeated measures ANOVA were used to compare body weight, fat and carbohydrate oxidation, resting metabolic rate, and respiratory exchange ratio (RER) across menstrual phases. One-way repeated measures ANOVA were performed to compare fasting AG and active GLP-1 concentrations across menstrual phases. To calculate the relative change in hormones, data were normalized to baseline values and analyzed as change from fasting. To compare the changes of AG and active GLP-1, 4 x 3 repeated measures ANOVA (time x phase) were performed. Absolute AG and GLP-1 concentrations were also analyzed but the same results were found so for clarity only relative change data will be reported. Hormone concentrations were used to calculate area under the curve (AUC) using the trapezoidal method (59) and these AUC values were compared using one-way ANOVA across menstrual phases. Perceptions of appetite were also normalized to baseline and analyzed with 4 x 3 repeated measures ANOVA (time x phase). All tests were performed separately on oral-contraceptive users and naturally cycling females and similar trends were observed. Where significant main effects were found, post-hoc analysis using a Bonferroni correction for multiple comparisons was performed. Statistical significance was set to $p < 0.05$. All data are presented as mean±standard deviation.

Results

Participants. Participant body weight did not change (P=0.249) across menstrual phases (Table 1). Participants all reported regularly eating breakfast in the mornings (80±9 mm) and successfully replicated energy intake the day before all sessions (FP: 1442.8±540.7 kcal, OP: 1492.8±671.15, LP: 1582.6±314.7). Three participants were currently using tri-phasic oral contraception (Tri-cyclen lo, Janssen Pharmaceuticals, ONT, Canada) for >1 y and maintained usage throughout the study while the other 5 participants had not been using any form of hormonal birth control for >1 y prior to participation.

Table 1: Participant characteristics. *Note:* unlike letters represent significant differences (P<0.05).

	Follicular Phase	**Ovulatory Phase**	**Luteal Phase**
Session day	4.0±1.0	9.4±1.1	23.2±1.6
Body weight (kg)	63.9±15.3[a]	63.3±14.5[a]	63.4±15.2[a]
Body temperature (°C)	36.2±0.1[a]	36.2±0.1[a]	36.7±0.1[b]
E_2 ($pg \cdot mL^{-1}$)	26.92±15.58[a]	180.26±53.07[b]	98.08±17.67[c]
P_4 ($Ng \cdot mL^{-1}$)	1.39±1.48[a]	0.90±0.50[a]	43.05±14.79[b]

Body temperature indicated that ovulation had occurred in the non-contraception using females prior to the LP session (Table 1); body temperature was significantly elevated during the LP compared to FP (P=0.030) and OP (P=0.034). Moreover, fasting plasma E_2 and P_4 concentrations (Table 1) confirm proper timing of testing sessions and are in accordance with accepted concentrations for E_2 and P_4 during all menstrual phases (Eagle Biosciences, Inc., NH, USA). E_2 was elevated during the OP compared to both the FP (P=0.003) and LP (P=0.024); E_2

was also elevated in the LP compared to the FP (P=0.001). P_4 was elevated during the LP compared to both the FP (P=0.004) and OP (P=0.005); P_4 was similar during the FP and OP (P=0.306).

Appetite-Regulating Hormones. There was no difference in the fasting concentrations of AG across the menstrual phases (P=0.848, η_p^2=0.023). There was no effect of menstrual cycle phase (P=0.959, η_p^2=0.006) or phase x time interaction (P=0.433, η_p^2=0.126) on the post-prandial change in AG. There was a main effect of time (P=0.008, η_p^2=0.424) such that AG concentrations were decreased 30 (P=0.042), 60 (P=0.014), and 90 min post-prandially (P=0.034) compared to fasting (Figure 1). There was no difference (P=0.185, η_p^2=0.214) in the AG AUC.

Figure 1: (A) Relative change from baseline and (B) AUC of AG during each menstrual phase. No significant differences were found between phases. *Note:* * - indicates significantly lower relative to fasting (P=0.008); AG – acylated ghrelin; AUC – area under the curve. All data presented as mean±SD.

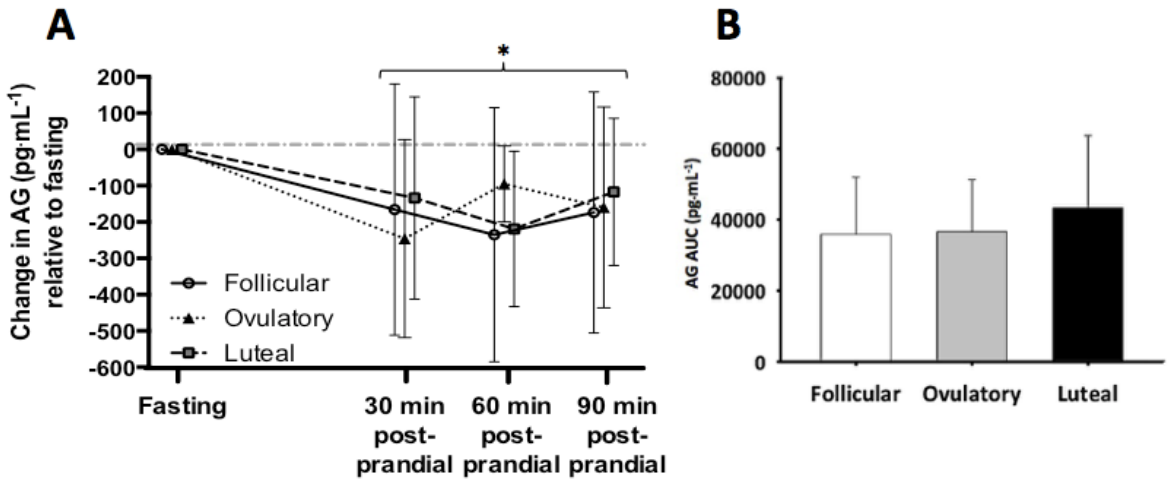
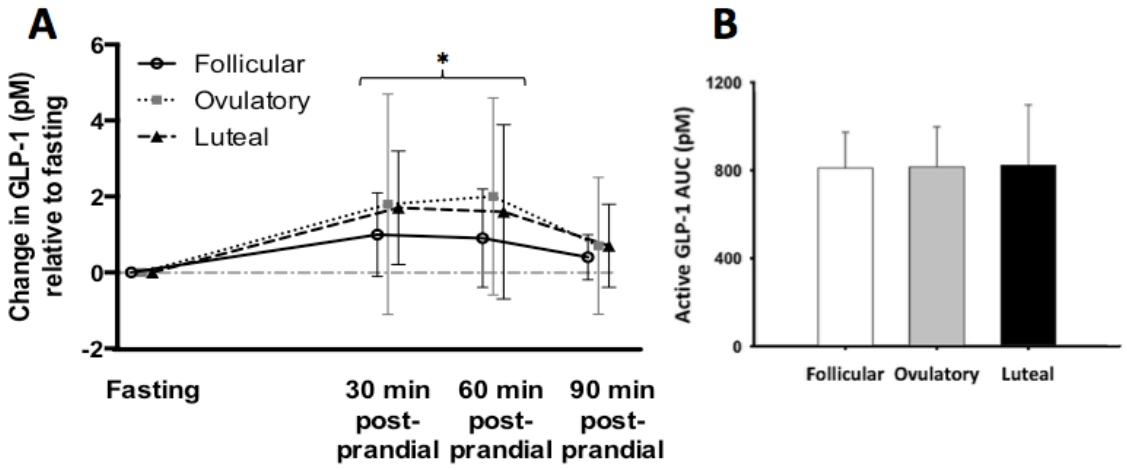


Figure 2: (A) Relative change from baseline and (B) AUC of active GLP-1 during each menstrual phase. No significant differences were found between phases. *Note:* * - indicates significantly elevated relative to fasting (P=0.027); AG – acylated ghrelin; AUC – area under the curve. All data presented as mean±SD.

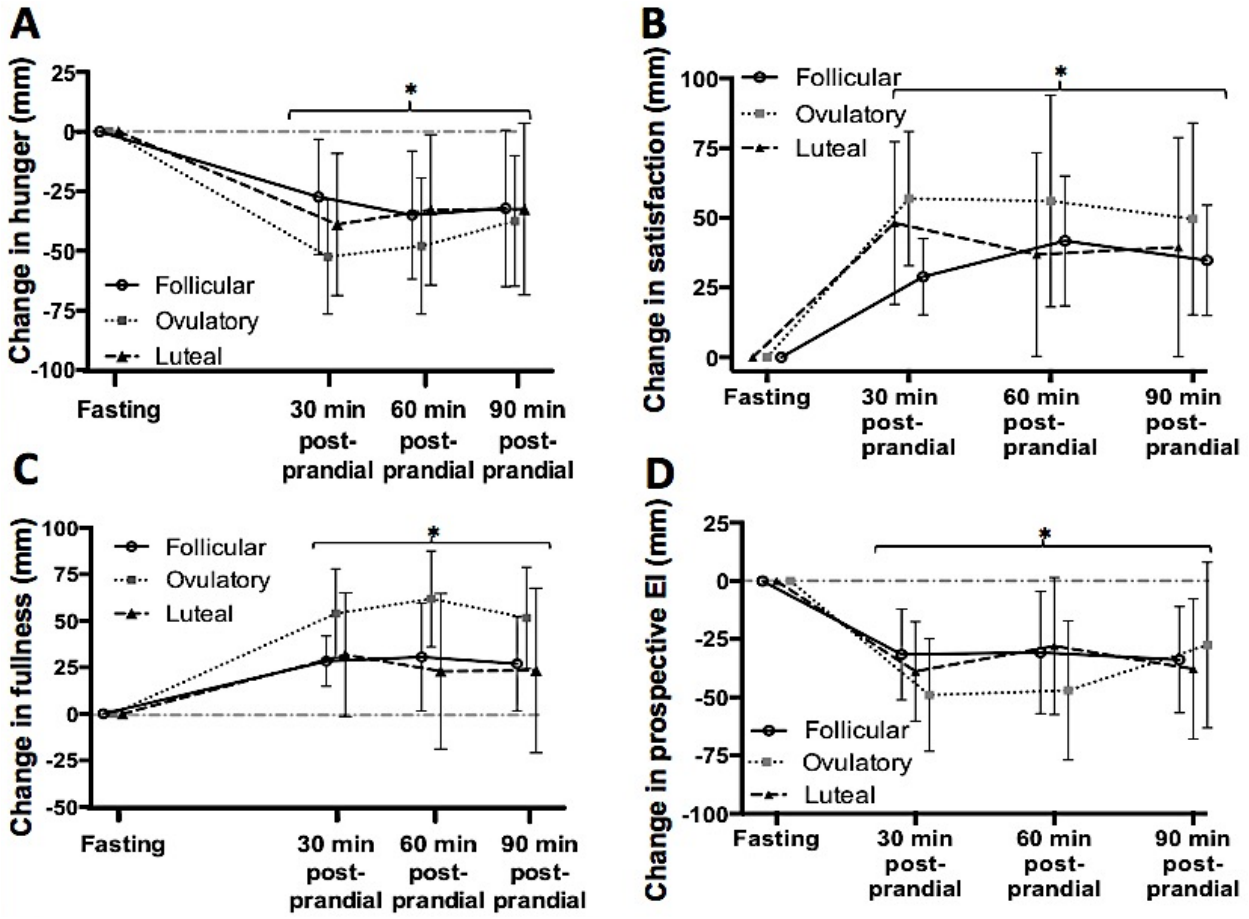


There was no difference in the fasting concentrations of active GLP-1 across the menstrual phases (P=0.567, η_p^2=0.051). There was no effect of menstrual cycle phase on the relative change in active GLP-1 from fasting (P=0.650, η_p^2=0.06) or a phase x time interaction (P=0.398, η_p^2=0.069). There was a main effect of time (P=0.027, η_p^2=0.483) where active GLP-1 was elevated 30 (P=0.021) and 60 min post-prandially (P=0.043), but not 90 min post-prandially (P=0.051) compared to fasting (Figure 2). There was no difference (P=0.605, η_p^2=0.069) in active GLP-1 AUC.

Appetite Perceptions. There was no effect of menstrual cycle phase (P=0.263, η_p^2=0.174) or phase x time interaction (P=0.241, η_p^2=0.179) on perceptions of hunger but there was a main effect of time (P=0.001, η_p^2=0.660) (Figure 3A). Perceived hunger was lower 30 (P=0.001), 60 (P=0.002), and 90 min post-prandially (P=0.012) compared to fasting. There was a main effect of phase (P=0.048, η_p^2=0.351) and time on perceived fullness (P<0.001, η_p^2=0.693), but no phase x time interaction (P=0.1, η_p^2=0.262). The main effect of phase showed that fullness was

greater during the OP compared to the FP (P=0.02) and approached significance in the LP (P=0.06). The main effect of time showed that fullness was greater 30 (P<0.001), 60 (P=0.001), and 90 min post-prandially (P=0.009) compared to fasting (Figure 3B). For satisfaction there was a main effect of time (P<0.001, η_p^2=0.784) but no effect of phase (P=0.263, η_p^2=0.174) or phase x time interaction (P=0.235, η_p^2=0.167). Satisfaction was increased 30 (P<0.001), 60 (P=0.001), and 90 min post-prandially (P=0.002) compared to fasting (Figure 3C).

Figure 3: Changes in (A) perceived hunger, (B) satisfaction, (C) fullness, and (D) prospective energy intake during each menstrual phase. Note: * - indicates significantly different than fasting (P<0.05). All data presented as mean±SD.



For prospective energy intake, there was no effect of menstrual cycle phase (P=0.725, η_p^2=0.045) or phase x time interaction (P=0.110, η_p^2=0.210) but there was a main effect of time (P<0.001, η_p^2=0.705) (Figure 3D). Prospective energy intake was decreased 30 (P<0.001), 60 (P=0.001), and 90 min post-prandially (P=0.002) compared to fasting (Figure 3D). There was no difference in anxiety or nausea between phases (P>0.05; data not shown).

Table 2: Resting metabolic rate, RER, and substrate oxidation. No significant differences were found.

	Follicular Phase	**Ovulatory Phase**	**Luteal Phase**
Resting metabolic rate (kcal)	1381±297	1399±330	1421±378
Respiratory exchange ratio	0.92±0.07	0.88±0.04	0.91±0.04
Carbohydrate oxidation ($g \cdot min^{-1}$)	0.17±0.05	0.14±0.04	0.16±0.04
Fat oxidation ($g \cdot min^{-1}$)	0.03±0.03	0.04±0.02	0.03±0.02

Resting Metabolic Rate, RER, and Substrate Oxidation. There was no effect of menstrual cycle phase on resting metabolic rate (P=0.961, η_p^2=0.006), RER (P=0.399, η_p^2=0.123), carbohydrate oxidation rate (P=0.603, η_p^2=0.07), or fat oxidation rate (P=0.485, η_p^2=0.098) (Table 2).

Discussion

The present study was designed to explore the effect of female menstrual cycle phase on appetite-regulating hormones, resting metabolic rate, and perceptions of appetite. We hypothesized that appetite-regulating hormones and perceived appetite would fluctuate throughout the menstrual cycle in conjunction with female sex hormones. The main findings of this project are: (a) menstrual cycle phase had no effect on AG or active GLP-1, (b) post-prandial

fullness was greatest during the OP, and (c) resting metabolic rate and substrate oxidation were unaffected by menstrual phase. These results indicate that AG and GLP-1 are not responsible for changes in energy intake across the menstrual cycle.

We did not observe any effect of menstrual cycle phase on the concentrations of AG, similar to past reports (57,58,60). Estradiol is believed to reduce energy intake by reducing the quantity of food consumed at each meal (13,61), suggesting that the post-prandial hormonal response to energy intake would be different in conjunction with varying E_2 concentrations (e.g. a more profound decrease in post-prandial AG during the OP, thereby reducing hunger and energy intake, compared to the LP). We are the first to measure the post-prandial change in AG across the menstrual cycle and our data do not support this idea, nor are what would be expected when considering the consistent fluctuations in energy intake across the menstrual cycle (9-12). Therefore, our data extend on past work to show that fluctuations in sex hormones across a typical menstrual cycle do not affect AG or the post-prandial change in AG.

Similar to AG, there was no difference in active GLP-1 across menstrual phases. Only one other report has examined the effect of menstrual phase on GLP-1 and found that total GLP-1 concentrations were lower in the FP compared to the LP because of slowed gastric emptying during the FP (24). In contrast, our current results found no differences in the active form of GLP-1, suggesting that active and total forms of GLP-1 might be differently affected by female sex hormones. However, our data suggest that active GLP-1 is unlikely to be altered by menstrual phase or female sex hormones and therefore unlikely to explain the previously observed changes in energy intake.

Interestingly, we did find that perceived fullness was greater during the OP compared to LP, although perceived hunger and prospective energy intake were similar. These findings could

be produced by the elevated E_2 concentrations observed during the OP phase and would be in line with the reduced energy intake from past literature. (10,11,44). Additionally, although statistically insignificant, 30 min post-prandial hunger appeared to be reduced to a greater extent during OP (-50%) compared to both the FP (-27%) and LP (-33%). To our knowledge this is the first study to investigate these perceptions across the menstrual cycle. Our similar hormonal profiles in conjunction with the different perceptions of appetite imply that central mechanisms could account for the changing energy intake across the menstrual cycle. Perhaps E_2 and P_4 influence neuronal control of energy intake within the arcuate nucleus (ARC) such that similar hormonal concentrations lead to different perceptions of appetite. But given that perceived hunger and fullness correlate with actual energy intake (31) the current differences warrant future investigation.

In light of the current data, it seems that production of AG and active GLP-1 are not responsible for the changes in energy intake that occur throughout the menstrual cycle. Indeed, our work supports that the sensitivity to these hormones is changing, as has been demonstrated for other appetite-regulating hormones, such as cholecystokinin (19), total ghrelin (7), and leptin (62,63). Sensitivity to gut hormones would likely occur within the ARC as it is responsible for integrating hormonal signals to control energy intake (27). Both NPY/AgRP and POMC/CART neurons within the ARC possess abundant E_2 receptors (62-65). Similarly, prolonged (28 d) P_4 treatment up-regulated NPY and down-regulated CART genes (both of which would be expected to stimulate EI) (66). Altered sensitivity would explain why we found similar circulating hormone concentrations but different perceptions of fullness across menstrual phases.

Finally, we observed no difference in substrate oxidation or RMR across menstrual phases. While previous studies demonstrated a slightly elevated RMR during the LP phase (39-

43), the present data illustrates no differences across phase. However, RMR can be increased from 2.5-11.5% during the LP compared to the FP, and the current data is within this range, despite being statistically insignificant. Similarly, fat oxidation rate was increased during the OP (0.04 $g \cdot min^{-1}$) compared to the FP and LP (both 0.028 $g \cdot min^{-1}$) as we would expect when E_2 concentrations are elevated (67), although this was also statistically insignificant. Our small sample size likely contributes to our inability to detect the small differences that occur across menstrual phases.

An important limitation to the current project is that we had participants arrive at 0800 and assessed appetite-regulating hormones over ~2.5 h although there are circadian rhythms associated with female sex hormones. Specifically, P_4 concentrations decrease throughout the day and E_2 concentrations might decrease post-prandially (68) meaning we may have missed peaks or nadirs in female sex hormones. Future work should extend on the current project by measuring at different times of day and sampling hormones over longer fasting and post-prandial time periods. Moreover, we fed participants identical test meals (29 $kJ \cdot kg^{-1}$ body weight). Considering the amount consumed at each meals is responsible for the changes in energy intake observed throughout the menstrual cycle (44) further research should test post-prandial hormonal responses to meals of various caloric content and even macronutrient composition. Additionally, we measured only two appetite-regulating hormones, but there is evidence that others could be important, including cholecystokinin (69), leptin (18), and insulin (15). We also had a relatively small sample size and all females were young and normal weight. We intentionally collected on normal-weight females as body composition can attenuate the post-prandial rise in GLP-1 and fall ghrelin (70). In light of the particular challenge overweight females have with managing

body weight and their propensity to progress into obesity future research will need to confirm the present findings in overweight and obese females.

Conclusion

The current project was designed to test the effects of female menstrual cycle phase on appetite-regulating hormones and perceived appetite. We show that fasting and post-prandial AG and active GLP-1 are unaffected by female menstrual cycle phase. Given the difficulty females experience with weight management it is important to continue exploring potential mechanisms of appetite and energy intake regulation. In light of accumulating research, future work should investigate the effects of menstrual cycle phase on neuronal control of appetite and energy intake and their sensitivity to circulating hormones since we show that gut-derived hormones are unaffected.

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Chapter 3

General Discussion

The current project was designed to examine whether the changes in estradiol and progesterone across the menstrual cycle are related to changes in appetite-regulating hormones and perceptions of appetite. Our results demonstrate that menstrual cycle phase has no effect on AG or active GLP-1 but fullness was greatest during the OP. These data show that the concentrations AG and active GLP-1 are not responsible for the fluctuating energy intake across the menstrual cycle and suggest other mechanisms are affecting appetite.

We did not observe any effect of menstrual cycle phase on the concentrations of AG, similar to past reports (1-3). Importantly the circulating concentrations in the current project are similar to those reported in other studies (4-6). In light of previous research showing that estradiol can influence the production of ghrelin it is possible that the estradiol fluctuations across a normal menstrual cycle are not great enough to affect ghrelin production. Indeed, the human study showing that estradiol affects ghrelin production was in amenorrheic females who express diminished estradiol and progesterone concentrations (7) making it difficult to extrapolate the results to a healthy eumenorrheic population. Based on the current AG data, to detect a 20% difference in AG with 80% power, we would require 152 participants, reflecting that any potential difference is small and unlikely to explain the changing energy intake. Finally, we performed Pearson correlations between fasting AG and estradiol concentrations to see if we missed any possible relationships. The only significant relationship was an inverse correlation between AG and estradiol during the LP ($r=-0.764$, $P=0.027$). This result is interesting but difficult to reconcile with the other results showing that AG concentrations did not differ across menstrual phases. There were no significant correlations between progesterone and AG. We found moderate correlations between AG and perceptions of hunger at 30 ($r=0.584$, $P=0.003$), 60 ($r=0.481$, $P=0.021$), and 90 min post-prandially ($r=0.647$, $P=0.001$), as would be expected based

off of past literature (8). Our current results support previous work demonstrating that sex hormone fluctuations across the menstrual cycle do not affect ghrelin making it unlikely to explain the changes in energy intake.

Only one previous study examined the effect of menstrual phase on GLP-1 and found that total GLP-1 concentrations were lower in the FP compared to the LP (9). Contrary to this we found no difference in the active forms of GLP-1. The reasons for our conflicting results are unclear, but participants in the previous study were fed only 50 g glucose dissolved in 300 mL water (200 kcal) (9). Importantly, GLP-1 has important incretin effects, specifically inducing insulin secretion and glucose uptake by skeletal muscle and adipose tissue (10). Thus, a dose of glucose compared to a meal replacement, as in the current report, with ~0.2 g/kg of fat and ~0.5 g/kg protein content, might be expected to produce a different incretin hormonal response due to slower digestion and lower glycemic load. With the current GLP-1 data, to detect a 20% difference in active GLP-1 with 80% power, we would require 30 participants, suggesting that any difference is quite small. The current concentrations of active GLP-1 are slightly greater than that previously observed (11) but very few studies have examined this form of GLP-1. When we performed correlations between estradiol and active GLP-1, we observed significant correlations during the OP ($r=0.752$, $P=0.031$), LP ($r=0.816$, $P=0.013$), and approached significance during the FP ($r=0.696$, $P=0.055$). We found no significant correlations between GLP-1 and progesterone. We observed no significant correlations between active GLP-1 and perceptions of fullness. However, a past study found that GLP-1 only correlated with fullness during the late phase of satiety (60-180 min post-prandial), thus our more acute study might miss this relationship. Taken together, we show that active GLP-1 is unaffected by menstrual phase.

The only observed difference in the current report is that post-prandial fullness was greater during the OP compared to FP and approached significance in the LP. This result is interesting given that females consume less food during the OP (12). Importantly, this occurred in conjunction with increased estradiol concentrations, reflecting estradiol's anorexigenic properties (13,14). At the same time, although it was statistically insignificant, post-prandial hunger appeared to be suppressed to a greater extent during the OP compared to both the FP and LP. This result makes sense given the greater fullness, but nevertheless aligns with our hypothesis. However, there was no correlation between fasting estradiol concentrations and perceptions of hunger (r=-0.198, P=0.353) or fullness (r=-0.05, P=0.816). We saw no differences between perceptions of appetite during the LP, implying that progesterone is not producing an orexigenic effect during this phase. This was supported by no apparent correlations between progesterone and fasting hunger (r=0.046, P=0.832) or fullness (r=0.165, P=0.441). AG and GLP-1 concentrations were similar meaning these cannot explain the altered appetite we observed during the OP. However, altered sensitivity to appetite-regulating hormones, other appetite-regulating hormones such as CCK and leptin, or the circadian rhythms of estradiol and progesterone could all be contributing the current results.

The current report did not observe any difference in energy intake, as assessed by self-report food logs, across the menstrual cycle. When assessed as absolute kcal there was no difference in the average 48 h energy intake across sessions (P=0.850, η_p^2=0.010) (Table 2). We also found no difference in the consumption (grams) of protein (P=0.51, η_p^2=0.048), fat (0.357, η_p^2=0.071), or carbohydrate (0.283, η_p^2=0.086) (Table 2). When analyzed as kcal/kg body weight the same results were observed. This is opposed to numerous reports (12,15-17) demonstrating that energy intake increases by up to 90-500 kcal/day, or an increment of 3.9-

34.7%, during the LP compared to the FP (18). Although this difference is certainly capable of altering energy balance and body weight, it is a relatively small change to try and detect with food logs. People regularly underreport energy intake by ~25-50% (19) making it unsurprising that we were unable to detect a change that may be as little as 3%. Unfortunately, adherence to food reporting was poor (only 4 of 8 participants completed all 3 phases) and there was evidence of under reporting for the current report forcing us to remove this data from the manuscript. This is not uncommon as up to 72% of females underreport food intake (20,21) by as much as ~25% (20,21,22). Fluctuations in energy intake during the menstrual cycle are generally accepted so our current results are very likely due to our collection methods.

We did not detect any differences in resting metabolic rate or substrate oxidation despite numerous past reports demonstrating significant differences in energy expenditure across the menstrual cycle (23-25). Although the reason for this is unclear, our small sample size could contribute. As previously mentioned, the differences detected are generally quite small, ~8% (23), and are detected over long periods of time. Considering the current data (0.94±0.19 kcal/min), to detect an 8% difference in energy expenditure with 80% power, we would require 204 participants, affirming the small differences that exist across menstrual phases. However, because energy expenditure and energy deficits are important factors in the regulation of appetite-regulating hormones and perceived appetite (11,26), the current data does allow us to say that energy expenditure was not a confounding factor for hormonal concentrations or perceptions of appetite in the current report.

Limitations and Future Directions. It is important to recognize that this project explored only a small set of appetite-regulating hormones and that estradiol can influence other appetite-regulating hormones, such as CCK, leptin, and insulin. CCK is a satiety-inducing hormone and

there is evidence that estradiol increases its potency (14). It is not clear exactly how estradiol enhances the potency of CCK, but there is a site of interaction within the hypothalamus so it is possible that ARC neurons play a role (27). At the same time, leptin and insulin are important signals capable of reducing energy intake and are both influenced by estradiol (28). Much like CCK, the anorexigenic effect of leptin appears to be enhanced in the presence of estradiol. Eating was reduced more substantially in female rats compared to males when leptin was infused directly into their brains, an effect that was attributed to the anorexigenic potency of leptin being enhanced by estradiol (29). A potential mechanism for this is again the colocalization of leptin and estradiol receptors on NPY/AgRP and POMC/CART neurons (30). Finally, there are important sites of interaction between estradiol and insulin, including on NPY/AgRP and POMC neurons (31). Therefore, future projects will need to consider all of these possible interactions and test concentrations of hormones and neuropeptides across menstrual phases before we can conclusively say why energy intake consistently changes.

In light of the current data, it seems that the gut-derived hormones AG and active GLP-1 are not responsible for the changes in energy intake that occur throughout the menstrual cycle. However, changing sensitivity to these hormones still remains a viable explanation (29,32-34). Altered sensitivity to gut hormones likely occurs in the ARC given that both NPY/AgRP and POMC/CART neurons possess abundant E_2 receptors. Thus, E_2 might exerts it anorexigenic effect directly on the neurons that regulate energy intake (13,33,35,36). At the same time, up-regulated NPY and down-regulated CART genes (both of which would be expected to stimulate EI) were observed when female rats were treated with progesterone for 28 days (37). Taken together, these data implicate the ARC in the modulation of energy intake in response to fluctuations in female sex hormones throughout the menstrual cycle.

Historically the ARC was thought to be the key site for regulating energy intake, but it is now recognized that a broader neural network, including the fusiform, amygdala, hippocampus, and dorsal striatum contributes to the regulation of energy intake (32). Research is beginning to use brain-imaging techniques (e.g. fMRI) to explore how different brain regions respond to food intake or food images and has produced interesting results that are relevant to the current project. For instance, food images produced greater activation of lateral and dorsolateral prefrontal cortex, and parietal cortex in females compared to males (38). Moreover, food pictures activated the lateral orbitofrontal cortex, prefrontal cortex, hippocampus, amygdala, and fusiform of females scanned around ovulation, whereas only the fusiform was activated in the LP (32). These results likely reflect that numerous brain regions possess estrogen receptors (39) which could make them susceptible to the fluctuations in female sex hormones associated with the menstrual cycle. Taken together, these data suggest that the effects of female sex hormones are highly complex and can influence appetite and energy intake through various central mechanisms.

One important factor that must be addressed in future research on the menstrual cycle is how to accurately and easily monitor menstrual phase. A recent report used ultrasound scans of participants' ovaries every morning over a one month study (1) thereby affording great accuracy but high burden on researchers and participants. The current project used a combination of body temperature, similar to other reports (40,41), and predicted menstrual cycle days based off of menstrual history. Although this is a convenient method, the pre-ovulatory estradiol surge is particularly difficult to monitor and predict. Even the use of ovulation kits would not have been helpful as those measure the estradiol-induced surge in luteinizing hormone. Indeed, the menstrual cycle is inconsistent and can be perturbed by many factors (e.g. stress and exercise)

making it difficult to predict. Furthermore, up to 12% of menstrual cycles can be anovulatory in young healthy females (42). Anovulatory cycles are asymptomatic and produce diminished concentrations of both estradiol and progesterone during the LP (42) which will distort the conclusions of research on the menstrual cycle. Finally, although the current project divided the menstrual cycle into three phases, it is possible to do this differently. For instance, others divide it simply into pre- and post-menstrual phases (12) or into early follicular (days 1–4), late follicular (days 5–11), periovulation (days 12–15) and the luteal phase (days 16–28) (18). Together these issues exacerbate the complexity of conducting research on female physiology, especially research on the menstrual cycle.

Another potential confounding factor is our use of participants using tri-phasic hormonal contraceptives. Tri-phasic pills suppress endogenous hormone production (43) as can be observed by the low concentrations of estradiol and progesterone in the three participants using pills during the current project (see Appendix VI). However, tri-phasic pills, such as the one used by our participants, contain changing doses of an estrogen and progesterone and, so long as pills are taken as directed, produce a hormonal environment similar to a normal menstrual cycle (43). Indeed, authors of this study concluded that tri-phasic pills represent an ideal research tool for investigating the effects of ovarian hormones (43). To confirm that the current results were the same in non-oral contraceptive using participants we performed a 4 x 3 ANOVA (time x phase) on the relative chance in AG and active GLP-1 in only these five participants. As expected, there was no effect of phase or phase by time interaction on AG ($P=0.659$ $\eta_p^2=0.055$, $P=0.835$ $\eta_p^2=0.102$, respectively) or active GLP-1 ($P=0.313$ $\eta_p^2=0.252$, $P=0.547$ $\eta p^2=0.175$, respectively). Therefore, it is unlikely that the inclusion of participants using tri-phasic

contraception skewed our data and suggests that it could be beneficial for future research to include only tri-phasic pill users to monitor menstrual phase.

Conclusions. Until 1993, when the NIH mandated the inclusion of females in clinical research, most physiological research had been performed on males (44). Until this time, the primary function of female sex hormones was believed to be the creation of a hospitable environment for conception and fetal development (44). Gradually, it is becoming clear that females require unique research. This seems logical given that females experience unique hormonal fluctuations that likely have important physiological effects. Despite research beginning to appreciate the numerous functions of female sex hormones, when reviewing literature on the effects of exercise on appetite-regulating hormones there are 43 studies that looked at only male participants, 12 studies included males and females, five were females only, but of all the studies on females, only two controlled for menstrual phase; although they were only during early FP when both estradiol and progesterone are at their lowest concentrations (4,45). Together, these considerations make it important that research such as the current project continue to be performed.

We chose to focus on appetite-regulation given the changes in cravings and energy intake across the menstrual cycle (12,46). Ultimately, females are probably in a battle against biology and evolution. Given that small energy deficits can have detrimental effects on reproductive health and that females require a relatively high percentage of body fat to experience regular menstrual cycles, it is conceivable that numerous redundant mechanisms exist to ensure that females tightly maintain fat stores and energy intake. Indeed, central (e.g. various brain regions) and peripheral (e.g. numerous appetite-regulating hormones) mechanisms likely all contribute to the regulation of energy intake in females. The current report found that two gut-derived

hormones (AG and active GLP-1) are not responsible for the difference in energy intake across the menstrual cycle but we did find that perceptions of appetite differ across menstrual phases. We have proposed other mechanisms that warrant investigation to eventually contribute to our understanding of female weight management.

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Appendix I – Visual Analogue Scale

Participant Code: _____

Date: _____

Session: _____

Question 1: How often do you eat breakfast in the morning?

Never **Every morning**

_____

Question 2: How hungry are you right now?

Not hungry at all **Very hungry**

_____

Question 3: How full do you feel right now?

Not full at all **Very full**

_____

Question 4: How much do you think you could eat right now?

Nothing at all **A lot**

_____

Question 5: How anxious are you right now?

Not anxious at all **Very anxious**

_____

Question 6: How nauseated are you right now?

Not nauseated at all **Very nauseated**

_____

Appendix II – Daily Food Log

Instructions:

1. Record all food intake for a 3 day period (day before session, day of session, day after session)
2. Try to consume foods that you would typically eat as part of your regular diet.
3. Keep your recording sheets with you at all times. (Snacks are typically consumed unpredictably and, as a result, it is impossible to record them accurately unless your recording forms are nearby.)
4. Use a small food scale if you have one or standard-measuring devices (measuring cups, measuring spoons, etc) to record the quantities consumed, as accurately as possible. If you do not eat all of the item re-measure what's left and record the difference.
5. Record combination foods separately (i.e., hot dog, bun, and condiments) and include brand names of food items (list contents of homemade items) whenever possible.
6. For packaged items, use labels to determine quantities.

Example:

Time of Day (i.e. 8:15 am, 12:30 pm)	**Food Item** (include brand name if possible)	**Quantity** (i.e. g, mL, cups, etc.)	**Notes** (i.e. ingredients & amounts used if possible)
9:30 am	Eggs	2 whole	½ tsp salt, ½ cup cheese, ½ tsp butter
9:30 am	Egg whites	½ cup	-
10:15 am	Tropicana orange juice	1 cup	-
11:05 am	Apple	1 whole	-
1:50 pm	Dominos Pizza	4 slices	Pepperoni, mushroom, cheese
1:50 pm	Pepsi	500 ml	-

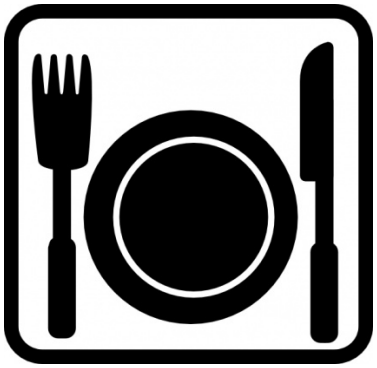
DAY 1 (day before session) Date: _____

Time of Day (i.e. 8:15 am, 12:30 pm)	**Food Item** (Include brand name if possible)	**Quantity** (i.e. g, mL, cups, etc.)	**Notes** (i.e. ingredients & amounts used if possible)

Appendix III – Study Recruitment Poster

FEMALES NEEDED FOR APPETITE REGULATION STUDY

(REB #4598)



What is Appetite Regulation?

- Various hormones control how much we eat
- The female menstrual cycle may alter these hormones

Purpose:

Determine whether the hormones associated with the menstrual cycle alters the hormones that control food intake

Study details:

- Each session involves 4 blood samples taken from the arm

- In order to participate you must be:
  - Female, 18-35 years of age
  - Regularly menstruating
  - Non-hormonal contraceptive user (or using tri-phasic pill, such as: Ortho Tri-Cyclen, Enpresse, and Ortho-Novum 7/7/7)
  - Healthy and exercise ≤3 per week
  - Non-smoker

- Time commitment:
  - 3 sessions of ~3 h (9 hrs total)
  - Sessions will be over 1 menstrual cycle (~1 month)

Benefits of participating:

- Learn and contribute to the better understanding of female physiology
- Three free meals!

If interested please contact:

Logan Townsend, M.Sc. student – **town9000@mylaurier.ca**

Appendix IV – Ethics

CONSENT TO PARTICIPATE IN RESEARCH

LETTER OF INFORMATION

Date:

Title of Study: **The Effect of Menstrual Phase on Energy Regulating Hormones in Response to Sprint-Interval Training (REB #4598)**

Dear _____:

You are being invited to participate in a research study conducted by Tom J. Hazell (PhD), Hashim Islam (BSc Kin), and Logan Townsend (BA Kin) from the Energy Metabolism Research Laboratory.

In order to participate it is important you are: a) between 18-30 year old, b) recreationally active <3/wk, c) healthy, d) normally menstruating, e) either using no form of hormonal contraceptive or using a tri-phasic pill as this project is designed to determine how these hormones influence appetite hormones.

PURPOSE OF THE STUDY

The primary purpose of this study is to examine the effects of acute sprint interval training on energy expenditure and hormones that regulate energy intake in women and how the menstrual phase may influence this in order to determine potential fat loss mechanisms.

PROCEDURES

This study requires you to visit the Energy Metabolism Research Laboratory 3 separate times, one for a familiarization session (<30 min) and then for 2 testing sessions (~4.5 h) for a total time commitment of ~9 hours. All experimental sessions (separated by 96 h) will include a standardized breakfast and 30 min exercise session (7 min warm-up, 18 min SIT exercise, and 5 min cool-down) followed by a 3 h post-exercise measure of gas exchange where participants rest comfortably and quietly in the laboratory (i.e. reading). Blood samples will also be drawn from the forearm pre- and post-exercise (4 samples total). Blood draws will be taken from the inner elbow while seated by a trained researcher; all equipment will be sterile one-time use. Blood draw site will be cleaned prior to each sample and researcher will wear clean gloves to ensure cleanliness. The exercise will involve traditional SIT characterized by 4 bouts of 30 sec "all-out" efforts followed by 4 min rest/active recovery (i.e. light walking). The exercise session includes only 2 min of work and 16 min of rest/recovery. You will also be asked several questions before and after exercise to determine your feelings of hunger and satiety. You will be asked to record dietary

intake the day before the trail, on trial day and the day after. You will also be required to give a detailed history of your menstrual cycle, including length of recent cycles, when you began menstruating, and if you have ever had irregular menstrual cycles.

POTENTIAL RISKS AND DISCOMFORTS

We appreciated that this study requires you to discuss highly personal and sensitive information and we will do our best to make you as comfortable as possible; should you feel uncomfortable at any point you are free to withdraw from the study. All information regarding the menstrual cycle is only used to ensure that our testing days are done at the appropriate times, this information will not be included in any publications or presentations and the participant will not be identifiable by the information the provide. There is a possibility of mild muscle soreness and/or fatigue typical of an exercise session. You may feel some discomfort due to the intensity of the training typical of strenuous physical exertion, as you are required to perform the training and testing sessions with maximal effort. Although phlebotomy is safe when done by certified and training individuals there is a small risk of bruising at the puncture site which can be reduced by keeping pressure on the site for several minutes after the needle is withdrawn. In some rare cases the vein may become inflamed after the sample is withdrawn however using a warm compress can alleviate this. There is a small risk of infection any time the skin is broken however this rarely occurs when equipment is properly sterilized and disposed of. Some people may also experience light-headedness if they are uncomfortable with needles and if this occurs the experiment will be terminated immediately. The risk of falling if this occurs is minimum as the participant will be seated in a secure phlebotomy chair.

POTENTIAL BENEFITS TO SUBJECTS AND/OR SOCIETY

The potential benefits of your participation in this study include an improvement in your exercise capacity using a novel, time-efficient training modality as well gain a better understanding of your cardiorespiratory fitness. Results from this study will further our understanding of high-intensity interval training and its effects on energy expenditure as well as participant's feelings towards these modifications of this intense form of training.

CONFIDENTIALITY

All information obtained in connection with this study will be de-identified. All contact information is collected and stored on a master list in a password-protected file with access to only the study investigators. All participants will be assigned an arbitrary number to ensure anonymity. This study number will be used in all data collection files and mean data will be stored in a password protected file for comparison with future studies. Raw data will not be released to any other parties and all results will be collapsed before analysis. All blood samples will be stored in a secured location until analysis and subsequently destroyed.

PARTICIPATION AND WITHDRAWAL

Your participation in this research study is completely voluntary. If you are a student and volunteer to be in this study, you may withdraw at any time without any effect on your status at Wilfrid Laurier University. If you are not a student, you may withdraw at any time. You may also refuse to answer any questions you feel are inappropriate and still remain in the study. The investigators may withdraw you from this research if circumstances arise which warrant doing so (i.e. lack of effort during exercise

sessions, difficulty scheduling, repeatedly missing scheduled sessions, etc.). Please note that should you withdraw from the study all personal information and blood samples will be immediately destroyed.

FEEDBACK OF THE RESULTS OF THIS STUDY

If you would like a copy of a lay summary of the results please check the box below. The results from this study will be reported in general terms in the form of speech or writing that may be represented in manuscripts submitted for publication in scientific journals, or oral and/or poster presentations at scientific meetings, seminars, and/or conferences. We plan to publish this study in an academic journal. The information published in a journal or subsequent studies will not identify you in any way. Copies will be available upon request.

☐

SUBSEQUENT USE OF DATA

This de-identified data may be used in subsequent studies (with no link to your personal information). You will receive a copy of the consent form after it has been signed and do not waive any legal rights by signing it.

This letter is yours to keep. If you have any questions about this research project feel free to call:

Dr. Tom Hazell 519-884-1970 x3048

Further, if you have any questions about the conduct of this study or your rights as a research subject you may contact Dr. R. Basso, Research Ethics Board (REB) Chair (rbasso@wlu.ca / 519-884-0710 x4994).

Sincerely,

Logan Townsend (town9000@mylaurier.ca), MSc Student

Hashim Islam (isla9020@mylaurier.ca), MSc Student

Dr. Tom Hazell (thazell@wlu.ca), Assistant Professor

Department of Kinesiology and Physical Education, Wilfrid Laurier University, Waterloo, ON

Title of Study: **The Effect of Menstrual Phase on Energy Regulating Hormones in Response to Sprint-Interval Training (REB #3862)**

Consent Statement

Principal Investigators: Dr. Tom Hazell

I have read the accompanying "Letter of Information" and have had the nature of the study and procedures to be used explained to me. All of my questions have been answered to my satisfaction.

By signing below, I agree to participate in this study

NAME (please print): _____

SIGNATURE: _____

DATE: _____

NAME OF PERSON OBTAINING INFORMED CONSENT (please print):

_____

SIGNATURE OF PERSON OBTAINING INFORMED CONSENT:

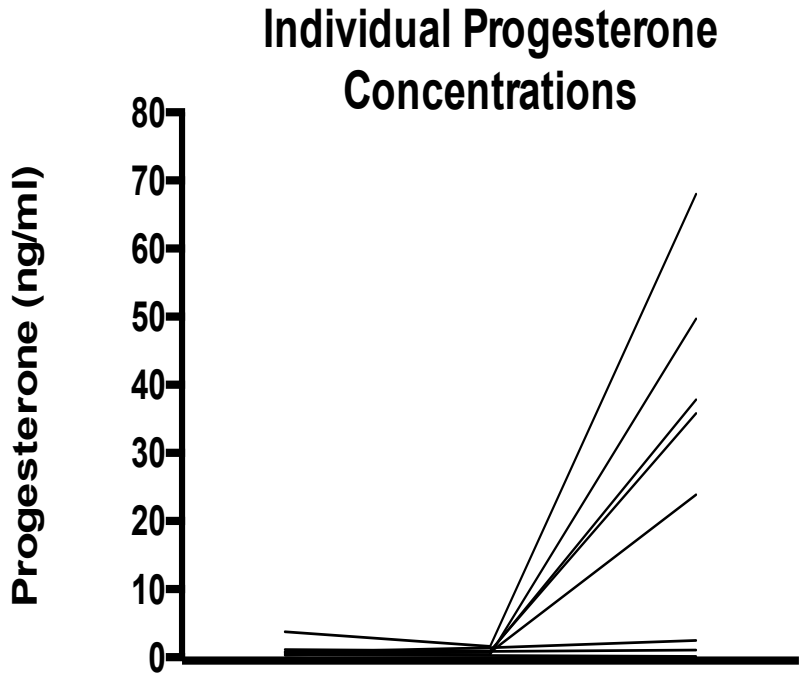
_____

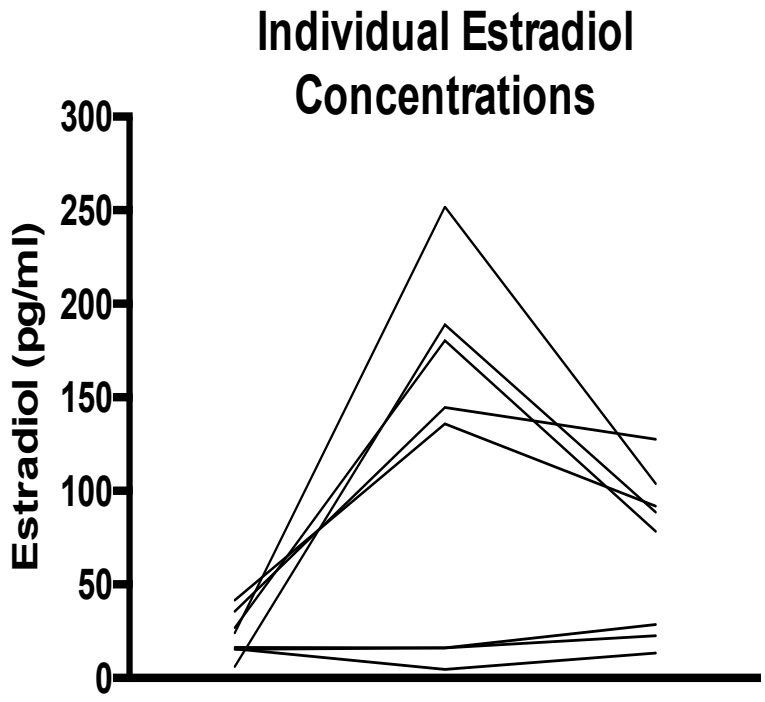
DATE: _____

Appendix V: Correlations

	Estradiol			Progesterone			AG			GLP-1		
	FP	OP	LP	FP	OP	LP	FP	OP	LP	FP	OP	LP
Estradiol	-	-	-	-	-	-	0.13	-0.41	-0.76*	0.69	0.75*	0.82*
Progesterone	-	-	-	-	-	-	-0.26	0.18	-0.46	0.19	0.42	0.54
AG	0.13	-0.41	-0.76*	-0.26	0.18	-0.46	-	-	-	-	-	-
GLP-1	0.69	0.75*	0.82*	0.19	0.42	0.54	-	-	-	-	-	-

Appendix VI: Individual Sex Hormone Concentrations





Appendix VII: Absolute Concentrations of AG and GLP-1

AG:

	Fasting	**30 min post-prandial**	**60 min post-prandial**	**90 min post-prandial**
Follicular	524.0±394.4	354.8±205.4	333.1±157.1	397.6±161.2
Ovulatory	576.1±422.9	378.5±163.1	343.7±165.8	416.4±221.7
Luteal	607.6±268.7	474.0±266.4	388.9±220.5	534.1±283.9

GLP-1:

	Fasting	**30 min post-prandial**	**60 min post-prandial**	**90 min post-prandial**
Follicular	8.0±1.9	9.1±2.6	8.9±2.3	8.4±1.9
Ovulatory	7.6±1.2	9.2±2.9	9.7±2.6	8.2±1.7
Luteal	7.9±1.9	9.9±3.1	9.6±3.9	8.7±2.7