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THE ROLE OF PEPTIDE YY AND GHRELIN IN REGULATING ENERGY HOMEOSTASIS AND REPRODUCTIVE FUNCTION IN YOUNG EXERCISING WOMEN

A Dissertation in
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by
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ABSTRACT

The primary purpose of this dissertation was to understand the physiological relevance of alterations in ghrelin and PYY in healthy populations of premenopausal women. The specific goals of this dissertation were to understand if PYY and ghrelin are involved in the long-term regulation of body weight in normal weight premenopausal healthy women (Study 1), to understand if alterations in PYY and ghrelin are related to reproductive function in premenopausal healthy women (Study 2 and 4), and to determine if college-aged women with different eating behavioral phenotypes, i.e., high vs normal dietary restraint, differ with respect to circulating concentrations of gastrointestinal hormones during and following a test meal (Study 3). Study 1 was designed to examine changes in fasting PYY and ghrelin in non-obese premenopausal women following an exercise and diet program with and without weight loss. We demonstrated that exercising women who lost weight increased ghrelin concentrations. Study 2 was designed to examine if elevations in 24 hour circulating ghrelin concentrations following a ~3 month exercise and diet program associated with diet- and exercise-induced weight loss are associated with a decrease in luteinizing hormone (LH) pulsatility in premenopausal women. We demonstrated that elevated ghrelin concentrations were associated with the suppression of LH pulsatility in premenopausal women. The purpose of Study 3 was to determine if college-aged women with different eating behavioral phenotypes, i.e., high vs normal dietary restraint, differ with respect to circulating concentrations of gastrointestinal hormones during and following a test meal. We demonstrated that PYY$_{3-36}$ concentrations were suppressed in the women with high dietary restraint compared to the women with normal dietary restraint, indicating low PYY$_{3-36}$ concentrations following a
test meal may be indicative of a blunted appetite reduction after a meal and, over time, lead to an increased risk of weight gain. The purpose of Study 4 was to determine if ghrelin and PYY are associated with the recovery of menses in women with exercise associated menstrual cycle disturbances (EAMD). We demonstrated that women with EAMD who experienced menstrual recovery experience significant decreases in fasting ghrelin concentrations compared to women with EAMD who did not resume menses. We speculate that decreases in ghrelin may be necessary, likely in conjunction with changes in other metabolic hormones, for exercising women with EAMD, to recover menstrual function. Overall, ghrelin is likely an important signal involved the regulation of the hypothalamic pituitary ovarian axis in premenopausal women, while PYY is related to eating behavior phenotypes after a meal.
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CHAPTER I

INTRODUCTION

Regular exercise is important for general health; however, many physically active women are at high risk for menstrual disturbances (1, 2). There is a cohort of physically active women who do not consume sufficient calories to match their energy expenditure requirements resulting in an energy deficit and, in many cases, ovarian suppression (3). The activity can be associated with exercise, but can also include occupational work and military training or combat. Ovarian suppression and energy deficiency have been associated with clinical sequelae including infertility, skeletal demineralization and stress fractures (4, 5).

Ghrelin and peptide YY (PYY) are affected by alterations in energy homeostasis; ghrelin being a marker of hunger (6) and PYY a marker of satiety. Ghrelin and PYY interact with leptin and insulin to regulate energy balance and provide peripheral signals to the central nervous system to regulate appetite, and short term food intake (7). Peripherally produced ghrelin is primarily secreted by distinct endocrine cells of the stomach and gastrointestinal tract and regulates the release of neuropeptide Y (NPY) and agouti-related protein (AgRP) centrally in the arcuate nucleus causing an increase in hunger (6, 8). While PYY is secreted from the intestine and also has ability to act centrally, PYY has the opposite effect of ghrelin, inhibiting NPY and activating the pro-opiomelanocortin neurons also centrally located in the arcuate nucleus to trigger satiety (9, 10). Interestingly, both PYY and ghrelin have been identified as peripheral signals of energy status that may modulate central hypothalamic factors associated with reproductive function (11-18).
II. Study One

Prospective studies that employ exercise and or weight loss interventions offer valuable insights into the role of fasting ghrelin and PYY. Our laboratory has previously demonstrated that fasting total ghrelin is elevated in healthy non-obese normally menstruating women following an exercise and diet program leading to weight loss but remains unchanged in subjects who exercised but did not lose weight (19). Fasting PYY has been investigated in obese populations following weight loss (20-23), but the results have been inconsistent and thus difficult to interpret. For example, fasting total PYY concentrations are elevated in obese children (20) and adolescents (21) after exercise training and weight loss, respectively. In contrast, Pfluger et al. (22) demonstrated a 30% decrease in fasting total PYY concentrations in obese patients after participating in an 8 week weight loss program. Similarly, Lien et al. (23) also demonstrated a decrease in fasting total PYY concentrations after 12 months of a weight loss intervention in obese adults. Clearly more research is necessary to understand the physiological relevance of alterations in fasting total PYY in both clinical and healthy populations. No studies to date have evaluated fasting total PYY concentrations before and after weight loss in healthy non-obese subjects and thus the role of PYY in the maintenance of long term energy balance in the absence of pathophysiology is unknown. Alterations in fasting total PYY after weight loss in healthy individuals may have long-term consequences that impact weight maintenance. Moreover, the relationship between alterations in PYY and other gastrointestinal peptides may help to elucidate a greater understanding of the complex physiology of energy homeostasis. This project will inform us of the role of PYY in long term energy balance in healthy non-obese women following an exercise and diet program designed to induce weight loss.
Aim 1: To determine the long-term changes in fasting total PYY concentrations in non-obese premenopausal women following a 3 month exercise and diet program leading to weight loss, and to determine if chronic exercise leads to changes in circulating total PYY in the absence of weight loss.

**Hypothesis 1i:** Women in the exercise and diet program that lose weight (>2.0kg) will have suppressed fasting total PYY concentrations compared to the weight-stable groups.

**Hypothesis 1ii:** Exercise in the absence of weight loss, will not have a significant effect on fasting total PYY, i.e., the exercising weight-stable group will have similar fasting total PYY concentrations compared to the non-exercising control group.

**Rationale 1i:** To date, studies of obese adults have demonstrated a decrease in fasting PYY concentrations (22, 23). Pfluger et al. (22) demonstrated a 30% decrease in fasting total PYY concentrations in obese patients who lost 1.9kg (5.4%) body mass, while, Lien et al. (23) also demonstrated a decrease in fasting total PYY concentrations in obese adults after a much longer weight loss intervention (12 months) with a much more dramatic weight loss of 6.3kg. Clearly, more research is necessary to understand the physiological relevance of alterations in fasting total PYY in both clinical and healthy populations. Since studies of obese adults suggest a decrease in total PYY concentrations following weight loss, we suggest that PYY concentrations will decrease following weight loss in non-obese women consistent with a possible role of PYY in the long term
regulation of energy balance. PYY may be an indicator of an energy deficiency, acting through the arcuate nucleus, inhibiting NPY and activating the pro-opiomelanocortin neurons, to regulate long-term energy homeostasis (9). To this end, alterations in PYY following weight loss may be related to changes in other energy homeostasis.

Ghrelin, a more established metabolic marker with potent orexigenic effects (24, 25), has been shown to respond to weight loss in healthy weight women (19). We have previously demonstrated that fasting total ghrelin is elevated in healthy non-obese women following an exercise and diet program leading to weight loss but remains unchanged in subjects who exercised but did not lose weight (19). The relationship between alterations in PYY and ghrelin may play a role in weight loss and weight maintenance in healthy populations. Riediger et al. (26) demonstrated that PYY can inhibit ghrelin neurons, indicating that PYY concentrations may be important when evaluating changes in ghrelin concentrations. Alterations in fasting total ghrelin and PYY after weight loss in healthy individuals may have long-term consequences that impact weight maintenance, specifically the relationship between ghrelin and PYY. We hypothesize that while ghrelin is elevated after weight loss, PYY will be suppressed, leading to an increase in hunger and making weight maintenance following weight loss difficult.

**Rationale 1ii:** To date studies have indicated that PYY is involved in the long-term regulation of energy balance and will be altered during energy deficiency situations, i.e., weight-loss secondary to caloric restriction or exercise (20-23). Only one study to date (21) has evaluated the effect of a long-term exercise program on PYY concentrations. However, this study was conducted in a population of overweight adolescents with no
control group. However, if exercise occurs during an adequate energy environment, i.e., during a weight stable scenario, PYY will not be altered. Exercise in the absence of weight loss will not have a significant effect on fasting total PYY, as energy balance is not markedly impacted.

**Expected Findings:** It is anticipated that adult women who lose weight following a three month exercise and diet program will have suppressed fasting total PYY concentrations compared to all the weight-stable groups (both the weight-stable control group and the non-exercising control group). We also expect that since both the exercising weight-stable group and the non-exercising control group will remain weight stable that that both groups will have similar fasting total PYY concentrations. These expected findings are in agreement with two studies (22, 23) of obese adults that demonstrated suppressed fasting total PYY following a weight loss program.

**Statistical Approaches for Aim 1:**

**Methods for data analysis**

Data screening will be conducted prior to analysis, involving outlier detection, and examination of variable distributions within each of the groups for normality. An ANOVA with repeated measures will be performed on PYY at pre- and post intervention. When main effects are detected, *post hoc* analyses will be performed using *t* tests employing the Bonferroni correction factor. Baseline measurements as well as change scores calculated from pre- and post study time points for other key variables, will be examined using a one-way ANOVA. When main effects are detected, post hoc analyses will be performed using the least-significant difference procedures. Pearson correlation
Coefficient analyses will be used to examine the relationship between changes in total PYY, total ghrelin and other variables of interest. In all analyses, \( P<0.05 \) will be considered statistically significant. Statistical analyses will be performed using SPSS software (version 16.0; SPSS Inc., Chicago, IL). Data will be reported as mean ± sd.

Sample Size Calculation

A power calculation was performed to determine sample sizes required to detect differences. For total PYY, sample size was based on the detection of a meaningful difference of 13.0 pg/ml and an SD of 7.55 pg/ml after a weight-loss program, based on previously published reports (22). In order to achieve 80% power for the total PYY analysis using a 0.05 level of significance, a sample size of 4 participants per group (total n= 12) will be required.

Data-set to be used

Data from the study titled “Bioenergetics”, a 3 month randomized controlled trial to examine the impact of caloric restriction combined with exercise on menstrual cyclicity and reproductive function in premenopausal, previously untrained women, will be used for the analysis.
III. Study Two

Women with anorexia nervosa and exercising women with amenorrhea experience both suppressed reproductive function and elevated ghrelin concentrations (27-31). There is evidence that ghrelin may be directly participating in the down regulation of reproductive function. Animal models have demonstrated a direct relationship between elevated ghrelin and reproductive suppression (12-14). Only one investigation to date has directly attempted to explore the relationship between elevated ghrelin and reproductive function in women (32). Messini and colleagues (32) examined the effect of a single ghrelin injection in normally menstruating women and failed to demonstrate any effect of the acute ghrelin injection on luteinizing hormone (LH). Other investigators (17, 18) have demonstrated that LH secretion is suppressed in men following a longer ghrelin administration indicating that a chronic elevation in ghrelin is necessary to elicit a suppression of LH pulses. Fasting ghrelin concentrations are chronically elevated in regularly menstruating non-obese premenopausal women following a 3 month exercise and diet program leading to weight loss (19, 33); however to date no investigations have evaluated if this chronic elevation in ghrelin is accompanied by a decrease in LH pulsatility.

Aim 2: To investigate if the elevations in circulating ghrelin following a ~3 month exercise and diet program leading to weight loss are associated with a decrease in LH pulsatility in non-obese premenopausal women.

Hypothesis 2: We hypothesize that the women in the energy deficient group will demonstrate a decrease in LH pulsatility compared to the no change in LH pulsatility in
control group. Additionally the increases in circulating ghrelin will be associated with a decrease in LH pulsatility.

**Rationale 2:** Energy restriction has previously been demonstrated to have negative consequences on reproduction. Short term manipulations of energy availability in sedentary women, i.e., carefully controlling energy intake and exercise expenditure, have consistently demonstrated energy availability to be related to changes in LH pulsatility (Williams 1985, Loucks 2003, Loucks 2006). Abou Heif (34) used an animal model to demonstrate that food restriction increases ghrelin concentrations in male rats and that the increases in ghrelin concentrations were inversely correlated with reproductive hormones including LH. To this end, many investigators have demonstrated that ghrelin administration suppresses LH pulsatility in men (17, 18), Rhesus monkeys (13, 35), male and female rats (14, 36), and decreases mean LH concentrations in female ruminants (37). Elevated ghrelin concentrations have been associated with decreases in LH in two pharmacological studies done in men (17, 18). Kluge and colleagues (17) demonstrated a decrease in LH pulse frequency and amplitude following four ghrelin injections (50µg) over a 12 hour period. Similarly, Lanfranco and colleagues (18) demonstrated that an 8 hour administration of acylated ghrelin decreased LH pulsatility. Both of these studies in men indicate that elevated levels of circulating ghrelin concentrations can negatively inhibit reproductive function by decreasing LH pulse frequency.

To date, the association between elevated ghrelin concentrations and reproductive function has not been demonstrated in women and we speculate that elevated ghrelin concentrations is one of the factors associated with suppressed reproductive function in
exercising women with amenorrhea. Metabolic and gastrointestinal hormones including ghrelin have the ability to cross the blood brain barrier and interact with NPY/AgRP and pro-opiomelanocortin (POMC)/cocaine- and amphetamine regulated transcript (CART) in the arcuate nucleus to affect appetite and additionally, are also hypothesized to affect the hypothalamic-pituitary-ovarian axis (11). A schematic of this hypothesis is shown in Figure 1.

We hypothesize that, in the current study, chronic increases in circulating ghrelin concentrations will decrease LH pulsatility; while the exact mechanism causing elevated ghrelin to down regulate LH secretion from the anterior pituitary is unknown, the animal literature suggests that elevated ghrelin causes alterations in gonadotropin-releasing hormone (GnRH) secretion from the GnRH neurons (14, 38). Lebrethon and colleagues (38) administered ghrelin to male rats and demonstrated a decrease in the GnRH interpulse interval, while Furuta and colleagues (14) suggest that since ghrelin injected in ovariectomized rats decreases only LH pulse frequency and not LH pulse amplitude, ghrelin presumably affects LH secretion at the level of the GnRH pulse generator. The ghrelin receptor, the growth hormone

Figure 1. The schematic displays that ghrelin, as well as leptin, insulin, and peptide YY (PYY3-36) all have the ability to cross the blood brain barrier and interact with neuropeptide Y (NPY) and agouti-related protein (AgRP) and pro-opiomelanocortin (POMC) and cocaine- and amphetamine regulated transcript (CART) in the arcuate nucleus to affect the hypothalamic-pituitary-ovarian axis.
secretagogue receptor, has been identified in the hypothalamic neurons including the arcuate nucleus and paraventricular nucleus (39). While ghrelin is not necessarily acting directly on the GnRH neurons, it is more likely acting on other neuropeptides that have also been demonstrated to regulate the GnRH pulse generator. Specifically, elevated ghrelin concentrations can cause alterations of neuropeptides including NPY, POMC, and kisspeptin (KiSS-1) (40, 41). Alterations in these neuropeptides then down regulate GnRH secretion leading to a downstream decrease in pituitary release of LH (40-43). A schematic of this hypothesis is shown in Figure 2.

**Expected Findings:** It is anticipated that the women in the energy deficient group will demonstrate a decrease in LH pulsatility compared to the control group. The control group will demonstrate no change in LH pulsatility. We also anticipate that increases in circulating ghrelin will occur following the 3 month exercise and diet program (19, 33),

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**Figure 2.** The schematic displays that ghrelin has the ability to cross the blood brain barrier and interact with KiSS-1, neuropeptide Y (NPY) and pro-opiomelanocortin (POMC) in the arcuate nucleus. These neuropeptides act directly or indirectly on the gonadotropin-releasing hormone (GnRH) neurons to down regulate GnRH secretion. A decrease in GnRH secretion decreased luteinizing hormone (LH) section.
additionally, we anticipate that these increases in ghrelin with will be associated with decreases in LH pulsatility. These expected findings are in agreement with two pharmacological studies done in men (17, 18) demonstrating that elevated ghrelin concentrations are associated with decreases in LH.

**Statistical Approaches for Aim 2:**

**Methods for data analysis**

The time series of the 24 hour LH concentrations will be analyzed for pulse frequency, peak amplitude, peak height, and 24 hour mean LH using a pulse detection algorithm Cluster (44). Data screening will be conducted prior to analysis, involving outlier detection, and examination of variable distributions within each of the groups for normality. Repeated-measures ANOVA will be used to determine whether changes in LH pulse frequency from Pre- to Post-intervention will be significantly different between the sedentary control group and the diet and exercise group. The change in LH following the intervention will be calculated. For comparisons of the changes between groups, independent t-tests will be employed. Pearson correlation coefficient analyses will be performed to examine relationships between LH and ghrelin. All analyses will be performed using SPSS software (version 16.0; SPSS, Inc., Chicago, IL). All data will be reported as mean ± standard error.
Sample Size Calculation

For LH pulse frequency, sample size was based on the detection of a meaningful
difference of 1.8 pulses/24 hours and an SD of 1.2 pulses/24 hours after a weight-loss
program, based on previously published reports demonstrating the effects of energy
availability on LH pulse frequency in women (45). In order to achieve 80% power for
the LH pulse frequency analysis using a 0.05 level of significance and a allocation ration
of N2/N1, a sample size of 6 is needed in the control group and 12 in the diet and
exercise group (total n= 18).

Data set to be used

Data from the study titled “Bioenergetics”, a 3 month randomized controlled trial to
examine the impact of caloric restriction combined with exercise on menstrual cyclicity
and reproductive function in premenopausal, previously untrained women, will be used
for the analysis.
IV. Study Three

Young college-aged women frequently display sub-clinical disordered eating behaviors, specifically some young women display an eating phenotype known as cognitive dietary restraint defined as an effort to consciously restrict food intake (46, 47). Cognitive restraint has been previously demonstrated to be positively correlated with fasting total ghrelin concentrations in non-obese men and women (48). Interestingly, Martins and colleagues (49) demonstrated that high cognitive restraint does not affect fasting or postprandial total PYY concentrations; rather elevated disinhibition is associated with suppressed circulating PYY concentrations following a meal, indicating that elevated PYY may be a sign of susceptibility to weight gain.

Alterations in reproductive function have been associated high cognitive dietary restraint (47, 50). Additionally, exercising women with amenorrhea also have high cognitive dietary restraint (51) in addition to elevated fasting elevated ghrelin (27) and PYY (51) concentrations. The alterations in fasting total PYY and ghrelin concentrations in exercising women with amenorrhea compared to exercising women with ovulatory menstrual cycles has been attributed to an energy deficient versus energy replete metabolic environment (3, 27-29, 51, 52). However, to date no studies have been conducted attempting to further understand the relationship between cognitive restraint and gastrointestinal hormones before and after a meal in young college aged women. Understanding the physiological cues, specifically gastrointestinal hormones, in young women with signs of sub-clinical disordered eating may help our understanding of eating behavioral phenotypes. Eating behavior phenotypes, such as women with high dietary cognitive restraint, may be associated with underlying biological characteristics such as
alterations in PYY and ghrelin that may explain why these women under eat. Understanding the relationship between eating attitudes and behaviors and gastrointestinal hormones may help to understand what may lead to under consumption of food in young college aged women.

**Aim 3:** To compare fasting and postprandial PYY\textsubscript{3-36} and active ghrelin concentrations in young women with high cognitive restraint to young women with normal cognitive restraint, and to compare fasting and postprandial PYY\textsubscript{3-36} and active ghrelin concentrations in young women with high disinhibition to young women with normal disinhibition.

**Hypothesis 3i:** Women with high cognitive restraint will have elevated ghrelin concentrations before and after a test meal compared to women with normal cognitive restraint, while cognitive restraint group will have no impact of PYY\textsubscript{3-36} concentrations before and after a test meal.

**Hypothesis 3ii:** Women with high disinhibition will have suppressed PYY\textsubscript{3-36} concentrations before and after a test meal compared to the women with normal disinhibition, while disinhibition group will have no impact of ghrelin concentrations.

**Rationale 3i:** To date ghrelin concentrations have not been evaluated after a meal in young women with high cognitive restraint who do not have eating disorders. Ghrelin concentrations, however, have been evaluated in women with anorexia and are
demonstrated to be chronically elevated (30, 31, 53). Nakahara et al. (Nakahara et al., 2007) previously demonstrated that ghrelin concentrations were elevated at baseline and following intake of a 400kcal standard meal in women with anorexia nervosa compared to normal weight controls. Similarly, following an oral glucose tolerance test, ghrelin concentrations were elevated in adolescent girls with anorexia nervosa compared to controls (53). To date, ghrelin concentrations following a meal in women with high cognitive restraint is unknown; however, Schur and colleagues (48) previously demonstrated that cognitive restraint was positively correlated with fasting total ghrelin concentrations in non-obese men and women and that fasting ghrelin concentrations were high in the high and intermediate cognitive restraint groups compared to the low cognitive restraint group. Interestingly, Borer and colleagues (54) have demonstrated that ghrelin concentrations respond rapidly to short-term changes in energy availability. We suggest that the behaviour associated with successful cognitive restraint leads to moderate changes in energy deficiency further leading to elevated ghrelin concentrations.

**Rationale 3ii:** Recently, Martins and colleagues (49) demonstrated that high cognitive restraint did not affect fasting or postprandial total PYY concentrations; rather elevated disinhibition was associated with suppressed circulating PYY concentrations following a meal. PYY concentrations are suppressed in obese patients before and after a meal (9, 20, 55, 56) and interestingly, obese patients demonstrate elevated disinhibition (57). We suggest that elevated disinhibition, weight gain, and elevated PYY are all interrelated and hypothesize that suppressed PYY concentrations may be a biological mechanism that promotes elevated disinhibition and drives appetite in young women.
**Expected Findings:** In agreement with Schur and colleagues (48), we expect that high cognitive restraint will be associated with elevated ghrelin and that we will demonstrate that women with high cognitive restraint will have elevated ghrelin concentrations before and after a test meal compared to women with normal cognitive restraint. However, since Martins and colleagues (49) demonstrated that high cognitive restraint did not affect fasting or postprandial total PYY concentrations we anticipate that cognitive restraint will have no impact of PYY$_{3-36}$ concentrations before and after the test meal.

We also anticipate that the women with high disinhibition will have suppressed PYY$_{3-36}$ concentrations before and after a test meal compared to the women with normal disinhibition, while disinhibition group will have no impact of ghrelin concentrations. These expected findings are in agreement with Martins and colleagues (49) who demonstrated that elevated disinhibition was associated with suppressed circulating PYY concentrations following a meal.

**Statistical Approaches for Aim 3:**

**Methods for data analysis**

Data screening will be conducted prior to analysis, involving outlier detection, and examination of variable distributions within each of the groups for normality. Repeated-measures ANOVA will be used to determine whether changes in active ghrelin concentrations will be significantly different following a meal in women with high cognitive restraint compared to women with normal cognitive restraint. Repeated-measures ANOVA will also be used to determine whether changes in PYY$_{3-36}$
concentrations will be significantly different following a meal in women with high disinhibition compared to women with normal disinhibition. The area under the curve following the meal will be calculated for both PYY\textsubscript{3-36} and active ghrelin. For comparisons of the changes in the AUC (PYY\textsubscript{3-36} and active ghrelin) between groups, independent t-tests will be employed. All analyses will be performed using SPSS software (version 16.0; SPSS, Inc., Chicago, IL). All data will be reported as mean ± standard error.

**Sample Size Calculation**

For PYY, sample size was based on the detection of a meaningful difference of 5pmol/L and an SD of 23pmol/L following a meal in women with high disinhibition compared to women with normal disinhibition, based on previously published reports (49). In order to achieve 80% power for the repeated measured analysis of PYY using a 0.05 level of significance, a sample size of 18 participants per group (total n= 36) will be required.

For ghrelin, sample size was based on the detection of a meaningful difference of 100pmol/l and an SD of 550pmol/L following a meal in women with high cognitive restraint compared to women with normal cognitive restrain, based on previously published reports (30). In order to achieve 80% power for the repeated measured analysis of ghrelin using a 0.05 level of significance, a sample size of 13 participants per group (total n= 26) will be required. However, a sample size of n=36 per group will provide a 92% power to test the hypothesis.
Data set to be used

Data from the study titled “Eating Behaviors Phenotypes” will be used for the analysis.

The purpose of this study was to examine the role of behavior and physiology on appetite regulation in young adult women.
V. Study Four

Both PYY and ghrelin have been identified as peripheral signals of energy status that may modulate central hypothalamic factors associated with reproductive function (12-18), i.e. the alterations in ghrelin and PYY observed in exercising women with amenorrhea may be involved in suppressing the hypothalamic-pituitary-ovarian axis and causing amenorrhea via mechanisms secondary to nutritional energy deficiency.

Increased caloric intake in women with amenorrhea is hypothesized to be an appropriate method of improving energy status and reversing amenorrhea (58, 59). This strategy is currently being tested in our laboratory as part of a large extramurally funded grant titled: Increased caloric intake to reverse energy deficiency in premenopausal exercising women: Impact on energy and bone status, and menstrual cyclicity. The primary objective of the overall project is to examine the impact of 12 months of increased caloric intake on menstrual cyclicity, estrogen status, metabolic indices of energy, and bone health in women with oligo/amenorrhea secondary to a chronic energy deficiency.

Increased caloric intake in exercising women (58, 59) and monkeys (60) with amenorrhea has been demonstrated to reverse amenorrhea; however, to date, investigators have not investigated whether PYY and ghrelin, acting as metabolic signals to the arcuate nucleus, up regulate reproduction and reverse amenorrhea during a period of increased caloric intake. This information will lead to a better understanding of the mechanisms causing hypothalamic amenorrhea and may provide information to formulate recommendations regarding the treatment of hypothalamic amenorrhea.

Aim 4: To determine if PYY or ghrelin are associated with a resumption of menses in exercising women with amenorrhea in response to increased caloric intake.
**Hypothesis 4i:** Exercising women with amenorrhea who resume menses will demonstrate a decrease in fasting ghrelin concentrations compared to exercising women who do not resume menses.

**Hypothesis 4ii:** Exercising women with amenorrhea who resume menses will not demonstrate a change in fasting PYY concentrations compared to exercising women who do not resume menses.

**Rationale 4i:** Ghrelin has been identified as peripheral signal of energy status that may modulate central hypothalamic factors associated with reproductive function (13). Suppressed ghrelin concentrations have been associated with weight gain in women with anorexia nervosa (30). Weight gain will likely occur in exercising women who increase calories and resume menses; the weight gained will likely cause ghrelin suppression similar to that seen in women with anorexia nervosa who gain weight (30).

We speculate that elevated ghrelin is one of the factors associated with suppressed reproductive function in exercising women with amenorrhea, and further, we suggest that ghrelin concentrations will decrease prior to the resumption of menses in exercising women with amenorrhea. Increases in caloric intake and the likely associated weight gain will precede the decrease in ghrelin concentrations. Ghrelin is secreted by endocrine cells of the stomach and an increase in food intake will lead to a decrease in secretion of ghrelin from these cells in the stomach (61). Decreases in circulating ghrelin concentrations cause a decrease in the hypothalamic release of NPY and AgRP which
have been demonstrated to cause a decrease in hunger intake (7). Additionally, we hypothesize, that in the current study decreases in circulating ghrelin concentrations in exercising women with amenorrhea will up regulate reproductive function leading to the resumption of menses. While the exact mechanism that causes changes in circulating ghrelin to be associated with the resumption of menses is unknown, the animal literature suggests that changes in ghrelin will cause alterations in GnRH (14, 38). Elevated ghrelin causes a decrease in GnRH indirectly through alterations of neuropeptides including NPY, POMC and KiSS-1 (40, 41), indicating that decreases in ghrelin concentrations will reverse these alterations and up regulate GnRH, leading to a downstream increase the in pituitary release of LH (40-43) and the resumption of menses. A schematic of this hypothesis is shown is Figure 3.

**Figure 3.** The schematic displays that ghrelin has the ability to cross the blood brain barrier and interact with KiSS-1, neuropeptide Y (NPY) and pro-opiomelanocortin (POMC) in the arcuate nucleus. These neuropeptides act directly or indirectly on the gonadotropin-releasing hormone (GnRH) neurons to down regulate GnRH secretion. An increase in GnRH secretion increases luteinizing hormone (LH) section and leads to the resumption of menses.
**Rationale 4ii:** Elevated PYY concentrations have been speculated to modulate central hypothalamic factors associated with reproductive function (15, 16). PYY is elevated in exercising women with amenorrhea (51, 52), and suppressed PYY concentrations have been associated with weight gain in women with anorexia nervosa (30). However, we (Scheid et al. 2010, unpublished) recently demonstrated that weight loss in a normal weight population did not cause a change in PYY concentrations. Since exercising women with amenorrhea are normal weight, we hypothesize that weight gain in a normal weight population will not cause a decrease in PYY concentrations unlike that observed in anorexic women.

To this end, we speculate that PYY may be genetically predetermined as opposed to related to alterations in energy availability or weight loss (62). Suppressed PYY concentrations are observed in specific populations of obese patients (22, 23) and Boey et al. (63) suggested that suppressed fasting PYY concentrations may indicate a genetic predisposition that is associated with obesity and type 2 diabetes. Specifically, suppressed fasting PYY concentrations are observed in females with first-degree relatives who have type 2 diabetes (63). Moreover, it has been demonstrated in animal models that PYY null mice developed obesity (64) and also had elevated insulin secretion in response to glucose (63), indicating that a proportion of PYY secretion may be predetermined by genetic factors.

**Expected Findings:** Since Nakahara et al. (30) has demonstrated that ghrelin concentrations decrease following weight gain in women with anorexia nervosa and since, ghrelin concentrations are tightly regulated with changes in energy availability (54, 65), we anticipate that decreases in fasting circulating ghrelin concentrations will likely
occur in exercising women who increase calories and resume menses. While suppressed PYY concentrations have been associated weight gain in women with anorexia nervosa (30). However, we (Scheid et al. 2010, unpublished) recently demonstrated that weight loss in a normal weight population did not cause a change in PYY concentrations. Since exercising women with amenorrhea are normal weight, we do not anticipate that weight gain in a normal weight population will cause a decrease in PYY concentrations.

**Statistical Approaches for Aim 4:**

**Methods for data analysis**

Data screening will be conducted prior to analysis, involving outlier detection, and examination of variable distributions within each of the groups for normality. An ANOVA with repeated measures will be performed to compare fasting ghrelin and fasting PYY concentration in the exercising women with amenorrhea who resume menses to the exercising women who do not resume menses. When main effects are detected, post hoc analyses will be performed using $t$ tests employing the Bonferroni correction factor. Baseline measurements as well as change scores calculated from pre- and post study time points for other key variables, will be examined using a one-way ANOVA. When main effects are detected, post hoc analyses will be performed using the least-significant difference procedures. Pearson correlation coefficient analyses will be used to examine the relationship between changes in total PYY, total ghrelin and other variables of interest. In all analyses, P<0.05 will be considered statistically significant. Statistical analyses will be performed using SPSS software (version 16.0; SPSS Inc., Chicago, IL). All data will be reported as mean ± standard error.
Sample Size Calculation

A power calculation was performed to determine sample sizes required to detect differences. For fasting total ghrelin concentrations, sample size was based on the detection of a meaningful difference of 113pmol/l and an SD of 382pmol/l after a weight gain program, based on previously published reports in women with anorexia nervosa (30). In order to achieve 80% power for the total ghrelin analysis using a 0.05 level of significance, a sample size of 11 participants per group (total n= 22) will be required, for the analysis.

A power calculation was performed to determine sample sizes required to detect differences. For fasting total PYY concentrations, sample size was based on the detection of a meaningful difference of 6pmol/l and an SD of 28pmol/l after a weight gain program, based on previously published reports in women with anorexia nervosa (30). In order to achieve 80% power for the total PYY analysis using a 0.05 level of significance, a sample size of 15 participants per group (total n= 30) will be required, for the analysis.

Data-set to be used

Data from the study titled “REFUEL Active Women’s Study II: The Impact of Increased Caloric Intake on Bone Health and Menstrual Cyclicity in Energy Deficient Exercising Women” will be used for the analysis. The purpose of REFUEL was to test the effectiveness of 12 months of increased food intake to improve bone health and restore
menstrual cycles in women with menstrual disturbances who exercise regularly and have a chronic energy deficit.
References


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CHAPTER II part 1


INTRODUCTION

Since the passage of Title IX in the United States, opportunities for women to participate in physical activity (PA) and sport have dramatically increased. One of the earliest reports of menstrual irregularity in female athletes was published in 1962, and since then numerous studies have confirmed this finding in a wide range of athletes across a spectrum of various sports (1-4). Since then, the prevalence of menstrual disturbances has been well described in both recreational runners and athletes (5, 6).

One of the earliest investigators to propose a mechanism to explain the high prevalence of menstrual disorders in athletes was Dr. Michelle Warren suggested that “energy drain” or the energetic cost of exercise may play a role in the onset of menstrual cycle perturbations in physically active women and athletes (7). Since then, much has been published on the role of energy deficiency in the reapportioning of metabolic fuel away from reproduction and toward bodily functions such as cell maintenance, immune function and thermoregulation (8, 9). This paper explores the etiology of menstrual disturbances in exercising women and the role of gut peptides and adipocytokines in this mechanism.

I. Menstrual Disturbances in Physically Active Women

Menstrual cycle perturbations are often observed in women who participate in PA ranging from recreational to competitive, and modest to strenuous exercise training (5, 6).
Exercise-related menstrual disturbances are manifested along a continuum of severity ranging from subtle disturbances like luteal phase defects (LPD) and anovulation in asymptomatic cycles of regular length, to increasingly severe menstrual disturbances like oligomenorrhea, and amenorrhea (6, 10, 11). This continuum of menstrual disturbances is displayed in Figure 1. Amenorrhea represents the most extreme presentation of menstrual irregularity and is described as “functional hypothalamic amenorrhea” (FHA) when a disruption occurs at the level of the hypothalamus concomitant with exercise training (9, 12). FHA is characterized by chronic hypoestrogenism and is associated with severe clinical sequelae such as stress fractures, low bone mineral density and a potential increase in the risk of premature cardiovascular disease (13-16). Disordered eating, menstrual irregularities, and bone loss are interrelated medical conditions which comprise the syndrome known as the Female Athlete Triad (13). Figure 2 displays the interrelatedness of disordered eating, reproductive suppression and compromised bone health associated with the Female Athlete Triad.

The severe menstrual disturbances observed in physically active women and athletes include amenorrhea and oligomenorrhea. Amenorrhea has been defined as the absence of menstruation and can be further subdivided into two categories: primary and secondary. Secondary amenorrhea occurs sometime after menarche and is conservatively defined as no menses for a minimum of 3 months (6, 9). Amenorrhea in athletes is hypothalamic in origin, associated with reduced luteinizing hormone (LH) pulsatility, chronically suppressed ovarian steroids, and unaltered pituitary responsiveness to gonadotropin-releasing hormone (GnRH) in female athletes (12, 17). Primary amenorrhea is defined as the absence of menstruation by 15 y in girls with secondary sex
characteristics (18). Oligomenorrhea is defined by irregular and inconsistent menstrual cycles of 36 to 90 days in length (6), and in athletes, careful evaluation of the androgen environment should be considered to rule out irregular cycles associated with polycystic ovarian syndrome (PCOS) per se (19). Since PCOS is the most common cause of infertility in women, it is not surprising that some athletes bring PCOS to the athletic environment where it fails to be diagnosed and since these women may present with oligomenorrhea or amenorrhea, they are perceived to be oligo/amenorrheic secondary to an energy deficit, as opposed to PCOS (19).

(A) Metabolic changes of women with energy related menstrual cycle disturbances. Bar graph of REE per kilogram of FFM (kcal/d of REE per kg of FFM), triiodothyronine (TT₃, nmol/L), ghrelin (pg/mL), and leptin (mg/L) in the sedentary and exercising women grouped by menstrual status: sedentary with ovulatory cycles (SedOv), exercising with ovulatory cycles (ExOv), exercising with inconsistent cycles (ExIncon), exercising with consistent anovulatory cycles (ExAnov), and exercising with amenorrhea (ExAmen). p<0.05, *ExAmen, ExAnov, ExIncon vs. SedOv; † ExAmen vs. ExOv; §ExAmen vs. SedOv, ExOv, ExIncon, ExAnov; and ○ExAmen, ExAnov, ExIncon, ExOv vs. SedOv. Results are expressed as mean ± SEM. (B) Composite graphs of menstrual status depicted by daily estrone 3-glucuronide (E1G, ng/mL) and pregnanediol 3-glucuronide (PdG, µg/mL) concentrations in the sedentary and exercising women grouped by menstrual status. The E1G and PdG data for SedOv, ExOv, and ExIncon groups are aligned by the day of the LH peak, defined as day 0. The anovulatory (ExAnov) and amenorrheic (ExAmen) subjects' E1G and PdG data are aligned by chronological day of daily urinary hormone collections. The number of days depicted for the amenorrheic subjects is the mean cycle length of the menstruating subjects. Results are expressed as mean ± SEM. Reprinted from De Souza et al. Fertility
Prevalence studies on amenorrhea in athletes have reported variable estimates ranging from 1 to 66% (1, 3, 4, 6, 20, 21). Reported prevalence rates in females athletes are much greater than those observed in the general population of non-athletic, college-aged women (22, 23) largely attributable to the emphasis on the achievement of thin and lean physiques which may require low body weights and body fat percentages in sports such as figure skating, ballet, long-distance running and gymnastics. The prevalence of primary amenorrhea in a group of young triathletes was recently reported to be as high as
40% (24). The prevalence of oligomenorrhea in athletes has reported to range from 9-40% (2, 3, 11, 25-28), which is higher than the prevalence rate observed in non-athletic women of 5-11% (23, 29).

The subtle menstrual disturbances observed in physically active women and athletes include anovulation and LPD. Anovulation is defined as the absence of an ovulatory event or LH peak in association with reduced estrogen levels and the absence of luteinization (5, 6, 30). LPD is defined as short when the luteal phase length is less than 10 days (30), or inadequate when the sum of the 3-day mid-luteal peak pregnanediol glucuronide (PdG) is less than 10 µg/mL and when the PdG peak concentration is below 5 µg/mL (5, 6, 30, 31). In women with LPD, there is inadequate luteinization to support implantation, since the latter is dependent on an adequate progestational effect (30).

Figure 1 displays the spectrum of menstrual disturbances observed in exercising women when described via daily hormone measures of urinary excretion of estone-1 glucuronide (E1G) and PdG.

LPD and anovulation represent the most common menstrual abnormality associated with exercising training and physical activity (5, 6). The prevalence of LPD in athletes is much greater than that observed when compared to non-active or sedentary women. In a 3-month observational study, the prevalence of LPD and anovulation was found to be 48 and 79%, respectively in recreationally active women (5). More recently, we have confirmed these high prevalence rates (6). Indeed, as many as 50% of 120 cycles evaluated in exercising women were abnormal (LPD or anovulation); whereas, only 4% of 48 cycles evaluated in sedentary women were abnormal (6). The previous percentage is of concern because recreationally active women and athletes may be characterized by a
menstrual disturbance without any clinical demonstration or symptoms of the
abnormality since these disturbances occur in the face of normal cycle lengths. Because
these abnormalities are inconspicuous, both clinicians and active women must be
educated in order to understand and prevent these problems.

II. The Etiology of Exercise-Related Menstrual Disturbances

Menstrual disturbances in physically active women have been casually linked to a energy
deficiency where caloric intake is inadequate for exercise energy expenditure resulting in
a suppressive effect on growth and reproduction (9). The suppression of growth and
reproductive function during conditions of a chronic energy deficiency is a well-
recognized energy-conserving strategy intended to preserve fuel for more vital bodily
processes, such as cellular maintenance, immune function and locomotion (9, 32).
Reproduction, in particular, is a physiologically costly process that requires significant
energy reserves in order to sustain ovulatory function and adequate luteinization (33).

In exercising women, the evidence for a role of energy deficiency in the
modulation of reproductive function has been generated from observational and cross-
sectional studies of athletes and exercising women, and from prospective studies utilizing
both short- and long-term designs. Evidence from observational and cross-sectional
studies include data that demonstrate that exercising women with varying severity of
menstrual disturbances are discriminated from each other based on their metabolic profile
(8, 34, 35). Indeed, we examined the evidence for alterations in resting energy
expenditure (REE) and metabolic hormones in exercising women with menstrual
disturbances ranging from subtle disturbances like LPD and anovulation to severe
disturbances like amenorrhea (8). We observed adaptations consistent with chronic energy deficiency, including reductions in REE and total triiodothyronine (TT3) and elevations in ghrelin concentrations (8). Moreover, these alterations were observed across the continuum of clinical menstrual disturbances in a dose-response manner consistent with the severity of the menstrual dysfunction observed. In a study of 49 premenopausal women, De Souza et al. (8) demonstrated that the abovementioned energy deficiency increases in severity across the continuum of menstrual cycle disturbances such that the exercising women with amenorrhea, the most severe menstrual disturbance, had the lowest REE relative to fat free mass, the lowest total TT3 concentrations, and the highest ghrelin concentrations compared to the exercising women with subtle menstrual disturbances. The results of the study by De Souza et al. (8) are displayed in Figure 1. Figure 3 summarizes the mild to severe perturbations in metabolic status across the range of menstrual perturbations observed in exercising women, highlighting the fact that metabolic alterations increase in severity as the menstrual perturbation becomes more severe.

Prospective studies of short-term 4-5 day manipulations of food intake and exercise energy expenditure in sedentary women have also been demonstrated to alter metabolic profiles and LH pulsatility in a dose-response fashion at varying levels of energy availability (36-38). Prospective 2-3 month exercise training studies where energetic challenges were imposed in women (39, 40) by increasing energy expenditure and decreasing energy intake, demonstrated that these energetic challenges induced menstrual disturbances, including LPD, anovulation and delayed menses. Williams et al. (41, 42) also provided evidence for a causal relationship between energy availability and
menstrual cyclicity by inducing amenorrhea in monkeys during exercise training and then by reversing the amenorrhea and restoring ovulation in the monkeys by increasing food intake without moderating the energy expenditure. The restoration of ovulation in the amenorrheic monkeys was related to the volumes of calories consumed during refeeding such that the monkeys that ate the most calories resumed menses over the shortest interval (41). Confirmation that the suppression of reproductive function was coupled to energy-conserving mechanisms was the observation that alterations in total TT₃ was associated with both the induction and reversal of amenorrhea in the monkeys (41, 42).

![Diagram](image)

**Figure 3**

The schematic displays the metabolic hormones and substrates that are altered in exercising women with ovulatory menstrual cycles, compared to exercising women with luteal phase deficient menstrual cycles, and exercising women with amenorrhea. Reprinted from De Souza and Williams Hum Reprod Update 2004; 10:433-448, with permission.
Despite our understanding of the causal role of low energy availability in the induction of menstrual disturbances, appropriate practical guidelines of the optimal combination of dietary intake and exercise energy expenditure to maintain ovulation in exercising women remains undefined. Based on data in the experiments of Loucks et al. (38), it has been suggested that the threshold of energy available in physically active women must exceed 30 kcal/kg lean body mass/day to maintain normal LH pulsatility. More research is needed to confirm if this recommendation of energy availability translates to the maintenance of ovulatory function. Current efforts to review this issue and provide a set of guidelines for athletes and physically active women are underway by the Female Athlete Triad Coalition, an international consortium of professionals dedicated to optimizing the health of female athletes (www.femaleathletetriad.com).

III. Eating Behaviours, Gut Peptides, and Adipocytokines

In an effort to address a plausible mechanism for insufficient dietary intake in female athletes, much has been described regarding the physiological cues signaling food intake and psychological domains that control or modulate eating behavior. It is clear that in some cases where menstrual disturbances are secondary to an energy deficiency in athletic women, there is some form of disordered eating behavior that contributes to this condition (13, 43-45). The disordered eating behavior in athletes often presents as a high drive for thinness or as the conscious restriction of caloric intake, often referred to as dietary cognitive restraint (43, 46).
Although significant relationships between disordered eating behaviors have been related to menstrual disturbances in female athletes, it should not be assumed that every athlete has a psychological profile consistent with such pathology. It is logical to question whether the physiological cues for hunger and satiety in exercising women are distorted and are not appropriately coordinated to detect and meet exercise energy needs, or if some other cognitive cues that drive food intake are altered. In an effort to examine some of these issues, the roles of gut peptides and adipocytokines, important signals of hunger and satiety in exercising women are reviewed.

IIIa. Ghrelin

Ghrelin is a gastrointestinal hormone secreted by distinct endocrine cells of the stomach called X/A-like cells or ghrelin cells (47, 48). Peripherally-produced ghrelin can cross the blood brain barrier and regulate the release of neuropeptide Y (NPY) and agouti-related protein (AgRP) from the arcuate nucleus in the hypothalamus (49). Ghrelin activates the ghrelin receptors in the arcuate nucleus to upregulate the release of NPY and AgR, as well suppress the activation of the pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) leading to an increase in hunger and food intake (49, 50). Through these central mechanisms, ghrelin has been proposed to play a role in short-term energy homeostasis, and interestingly, ghrelin is the only circulating peripheral orexigenic hormone known to stimulate appetite (50). Ghrelin concentrations rise pre-prandially and fall postprandially in healthy individuals (51). The rise in ghrelin concentrations before a meal is a physiological signal for hunger and the body’s cue for meal initiation, and interestingly, the rise in ghrelin concentrations and hunger occurs independent of food and time of day cues (52). The rise in ghrelin concentrations across
the day is related to previous meal caloric intake, meaning that if subsequent meal caloric intake is low then an elevated ghrelin profile, associated with the next meal, will occur (53). Additionally, fasting ghrelin concentrations are demonstrated to be negatively correlated to 24 hour caloric intake (54). Ghrelin concentrations decrease following caloric intake (53), and the decrease in ghrelin release is related to the amount of calories ingested (53). Additionally, decreases in circulating ghrelin concentrations have been demonstrated to be related to carbohydrate intake (55) such that ghrelin concentrations decrease after consuming a carbohydrate rich meal, while protein and fat rich meals result in an increase in circulating ghrelin concentrations. Ghrelin infusions during a buffet meal are associated with an increase in food intake (56). Indeed, in 9 non-obese males and females, Wren et al. (56) intravenously infused ghrelin and demonstrated a 28% increase in caloric intake, indicating that the effects of circulating ghrelin concentrations are potent and can cause a large increase in appetite and food intake.

While ghrelin concentrations are tightly regulated by caloric intake, fasting circulating ghrelin concentrations are also negatively correlated to REE (54), indicating a role for ghrelin in regulating long-term energy homeostasis. Boer and colleagues (57) suggest that ghrelin concentrations respond rapidly to a negative energy balance, induced by either small meals or exercise. To this end, fasting ghrelin concentrations are elevated following weight loss due to diet and exercise in normal weight premenopausal women (53).

While weight loss is an example of an energy deficiency, i.e., energy intake is less then energy expenditure in order to achieve weight loss, anorexia nervosa is an example of a more severe, and chronic long-term energy deficiency. Anorexia nervosa is related to
a cognitive suppression of food intake leading to a chronic energy deficiency. Ghrelin is chronically elevated in women with anorexia nervosa (58-61) supporting a role for ghrelin as a hormone involved in long-term energy homeostasis. Total ghrelin secretion over 12 hours is elevated in women with anorexia nervosa compared to healthy controls and anorexic women fail to demonstrate a meal-induced reduction of ghrelin concentrations, indicating that ghrelin levels are chronically elevated in women with anorexia (58, 60). Since ghrelin concentrations are consistently elevated in energy deficient models, ghrelin may prove to be an important marker of energy deficiency and chronic undernutrition.

Another population demonstrating an energy deficiency is exercising women with FHA. Since the etiology of exercise-associated FHA is based on an energy deficiency and energy conservation, women with exercise-associated FHA also display evidence for elevations in fasting ghrelin concentrations (8, 35) and as previously described, other evidence of metabolic suppression including a decrease in REE, and suppression of total TT₃, insulin-like growth factor-1 and leptin concentrations and elevations in cortisol concentrations (8, 9). Whether ghrelin concentrations are chronically elevated remains to be determined since no meal studies have been published to date in this population. Despite the finding that fasting ghrelin concentrations are elevated in exercising women with FHA (8, 35, 62-64), increased appetite and/or increased food intake has not been reported in these women. Additionally, Boer and colleagues (57) suggest that ghrelin concentrations respond rapidly to a negative energy balance induced by exercise, and while ghrelin concentrations parallel energy status, elevated ghrelin concentrations do not induce hunger, supporting the finding that exercising women with FHA do not have
increased hunger despite their elevated fasting ghrelin concentration. Interestingly, a high drive for thinness has been reported in exercising women with FHA. Such women have elevated fasting ghrelin concentrations compared to women with a normal drive for thinness (65), indicating that if physiological hunger cues are elevated due to an energy deficiency, psychological cues, like a high drive for thinness, may act to suppress food intake.

Elevated ghrelin concentrations in exercising women with FHA and anorexia nervosa have been suggested to be related to a relative resistance to ghrelin (66). Ghrelin administration stimulates appetite in healthy and obese individuals (67) and ghrelin has been suggested as a potential treatment for women with anorexia nervosa to pharmacologically increase food intake by modulating hunger and to assist with weight gain. However, Miljic et al. (68) administered ghrelin to women with anorexia and failed to observe an increase in hunger (measured by visual analog scales). The study (68) demonstrated that ghrelin administration may not be an effective treatment to increase food intake in women with anorexia nervosa and supports the premise that women with anorexia nervosa are resistant to the orexigenic effects of ghrelin. The results from Miljic and colleagues (68) would likely translate to women with FHA and women, i.e., exercising women with FHA are also likely resistant, at least to some extent, to the effects of elevated ghrelin. Ghrelin resistance may be a physiological phenomenon; however it is likely that psychological components are also hindering the ability of ghrelin to simulate appetite. Similar to women with anorexia nervosa, the resistance to ghrelin in women with FHA may also be related to cognitive suppression of food intake.
observed in women with FHA with a high drive for thinness (65) and high dietary cognitive restraint (69).

Ghrelin and other metabolic and gastrointestinal hormones that are altered in women with amenorrhea may have a role in reproductive suppression (8, 35, 62). Many investigations have explored the relationship between metabolic alterations and reproduction (32, 42, 70, 71). Interestingly, metabolic and gastrointestinal hormones have the ability to cross the blood brain barrier and interact with NPY/AgRP and POMC/CART in the arcuate nucleus to affect appetite and additionally, are also hypothesized to affect the hypothalamic-pituitary-ovarian axis (72). A schematic of this hypothesis is shown in Figure 4. Elevated ghrelin concentrations in women with FHA may be involved in reproductive suppression; a direct relationship between elevated ghrelin and reproductive suppression has been shown in multiple animal models (71, 73, 74). Vulliemoz et al. (71) infused ghrelin in rhesus monkeys and demonstrated decreased LH pulse frequency, suggesting that GnRH activity is down-regulated by elevated circulating ghrelin concentrations. Messini et al. (75) investigated the effect of a single ghrelin injection in normally menstruating women and failed to demonstrate any effect of the acute ghrelin injection on LH, while other investigators (76, 77) have demonstrated that LH secretion is suppressed in men following a longer ghrelin administration. While many hormones are altered in women with FHA, ghrelin may be a hormone that, while attempting to regulate long-term energy homeostasis, contributes to the down regulation of GnRH, i.e., elevated ghrelin may contribute to the etiology of FHA.
IIIb. Peptide YY

Peptide YY (PYY) is a gastrointestinal peptide secreted from the endocrine L cells of the intestine in response to caloric intake (78-81). Similar to ghrelin, PYY can cross the blood brain barrier and act centrally at the level of the arcuate nucleus to contribute to eating behavior. While ghrelin simulates the releases of NPY and AgRP to increase hunger and food intake, PYY_{3-36}, the active form of PYY, activates the Y2 receptors to inhibit NPY and AgRP, as well activate POMC and CART, to decrease hunger and food intake.
intake (78, 82). Through these central mechanisms, PYY has a role in short-term energy homeostasis, and unique from ghrelin, causes a decrease in appetite. PYY concentrations rise in response to a caloric load after a meal (79, 81, 83) and stay elevated for several hours (80). Peak PYY concentrations are achieved in proportion to the amount of calories ingested (79, 84). While caloric intake is followed by an elevation in PYY concentration, two to three days of fasting suppresses PYY concentrations by 40-60% below baseline in lean men and women (85). PYY infusion in during a buffet meal is associated with a decrease in food intake (82). Batterham et al. (82) infused PYY₃₋₃₆ to 12 healthy non-obese men and women and demonstrated that caloric intake was decreased by 30%, indicating that the effects of PYY include large reductions in appetite and food intake.

Fasting PYY concentrations are tightly regulated by caloric intake and negatively correlated with BMI (86), suggesting a role for PYY in regulating long-term energy homeostasis. While the weight loss literature is inconsistent and difficult to interpret, obese children who lose weight have an increase in circulating PYY concentrations (87), and obese adults who lose weight have reported decreased PYY concentrations (88, 89). However, in women with anorexia nervosa, a population exhibiting a reduction in body weight and energy deficiency has consistently been observed to exhibit elevated fasting PYY concentrations (66, 88, 90). Similar to women with anorexia nervosa, exercising women with FHA secondary to an energy deficiency, also demonstrate elevated fasting PYY concentrations (62, 91). These data support the premise that specific energy deficient populations, including women with anorexia nervosa and exercising women with FHA, have elevated fasting PYY concentrations and PYY likely plays a role in regulating long-term energy homeostasis in these energy deficient models.
Interestingly, while fasting ghrelin, an orexigenic peptide, is elevated in women with FHA, PYY, an anorexigenic peptide, is paradoxically also elevated in women with anorexia nervosa and exercising women with FHA. Scheid et al. (62) reported that fasting concentrations of PYY are elevated in exercising women with FHA compared to both exercising women with ovulatory cycles and sedentary women with ovulatory cycles. Figure 5 summarizes the findings from Scheid and colleagues. Many other investigators have also demonstrated that fasting PYY concentrations are elevated in anorexic women (66, 88, 90). Elevated PYY concentrations are presumably related to an energy deficiency, as demonstrated by the negative correlations of PYY with BMI (86) and REE (62). The interpretation of these findings likely suggest that PYY could simply be a biomarker of long-term energy homeostasis related to energy deficiency, particularly in women with anorexia nervosa and exercising women with FHA. Elevated PYY has been suggested to facilitate decreased food intake secondary to the anorexigenic properties of PYY. Misra et al. (66) have suggested that elevated PYY may be involved in the
pathogenesis of anorexia nervosa and this may also play a role in the energy deficiency observed in exercising women with FHA (62). However, if PYY is indeed a physiological signal that is suppressing food intake, this signal may be compounding disordered eating behaviors in these women.

In addition to the role of ghrelin in regulating the hypothalamic-pituitary-ovarian axis, there is evidence to suggest that elevated PYY concentrations play a role in down regulating reproduction in women with anorexia nervosa and exercising women with FHA. Similar to ghrelin, PYY has the ability to cross the blood brain barrier and interact with NPY/AgRP and POMC/CART in the arcuate nucleus and can modulate appetite and food intake, and via these same mechanisms, is hypothesized to affect the hypothalamic-pituitary-ovarian axis (72) (Figure 4). Women with anorexia nervosa and FHA both experience suppressed reproductive function and elevated PYY concentrations (62, 66, 91). There is also evidence that PYY may be directly participating in the down regulation of reproductive function; however, to date, the evidence for this direct relationship has only been shown in animal models (92, 93). Investigations in Syrian hamsters have shown that infusions of PYY inhibit estrous (92). While Fernandez-Fernandez et al. (93) administered PYY₃-₃₆ to rats and observed a decrease in LH release, future research is needed to investigate if findings in these rodent models translate to human models, and if elevated circulating PYY concentrations in women with anorexia and exercising women with FHA is contributing to the down regulation of GnRH and LH pulsatility.

Lastly, elevated PYY concentrations may play a role in the uncoupled relationship between elevated ghrelin and food intake in energy deficient women. Exercising women with FHA demonstrate elevated ghrelin concentrations (8, 35, 62-64) and experience a
relative ghrelin resistance, i.e., women with FHA express behaviors including restricting food intake even in the presence of elevated physiological signals, like ghrelin, to increase food intake. As previously noted, PYY is elevated in exercising women with FHA and in women with anorexia nervosa (62, 91) and PYY has been shown to inhibit ghrelin neurons (94). As pointed out by Misra et al. (66), elevated PYY in the presence of elevated ghrelin may also cause a relative ghrelin resistance in these women. A comprehensive gastrointestinal profile in energy deficient women will be important to investigate to determine if there are physiological signals to explain the psychological behavior of suppressed food intake in energy deficient women.

IIIc. Leptin

Leptin is an adipocytokine that has been identified as a peripheral signal of energy status and is an important long-term regulator of food intake (95). Similar to ghrelin and PYY, leptin can cross the blood brain barrier and act on the arcuate nucleus in the hypothalamus (96). Leptin activates the leptin receptors to inhibit NPY and AgRP, as well activate POMC/CART to decrease food intake (96). Through these central mechanisms, ghrelin, leptin and PYY all participate in regulating energy homeostasis. Both animal (97, 98) and human (99) models have shown that a leptin deficiency can cause severe alterations in energy homeostasis leading to obesity, indicating that leptin is necessary for the long-term regulation of body weight. Leptin resistance is proposed to perpetuate obesity (100), i.e. a leptin resistance leads to a decrease in satiety, and a increase in food intake and weight gain and could play a role in obesity. Weight loss due to dieting decreases circulating leptin concentrations (101), indicating that a loss in fat mass is associated with a decrease in leptin secretion from adipose tissue. Although leptin
is correlated with body fat stores and weight loss (101, 102), two days of fasting suppresses fasting leptin concentrations independent of declines in fat mass (103). As such, leptin also acts as a short-term regulator of energy homeostasis since food intake stimulates leptin production while fasting inhibits leptin production (104).

Leptin is involved in the long-term regulation of energy homeostasis and is tightly correlated with body fat. Women with anorexia nervosa exhibiting chronic energy deficiency, have consistently been observed to exhibit suppressed fasting leptin concentrations (105). Audi et al. (106) cross-sectionally evaluated three groups of women with anorexia nervosa 1) underweight and amenorrheic, 2) weight-recovered and amenorrheic, and 3) weight-recovered and eumenorrheic and demonstrated that leptin concentration was suppressed in the women who were underweight, indicating that increasing body weight and leptin concentrations alone are not sufficient to reverse amenorrhea, but are likely important factors for the resumption of menses. To this end, Kopp et al. (107) demonstrated that a critical a leptin concentration or threshold was necessary for regular menses to occur in underweight women. Indeed, since leptin receptors have been identified at all levels of the hypothalamic-pituitary ovarian axis, and since there is in vivo and in vitro confirmation of an effect of leptin on the axis, strong support for a role of leptin in reproductive function is supported (108, 109).

Similar to women with anorexia nervosa, women with FHA also demonstrate suppressed leptin concentrations (110, 111). Miller et al. (110) demonstrated that women with FHA have suppressed leptin concentrations compared to age-, weight-, and body fat-matched eumenorrheic controls and that suppressed leptin concentrations may be related to low levels of caloric intake and/or suppressed fat intake in women with FHA compared
to the controls. Similarly, ballet dancers demonstrate a higher incidence of amenorrhea and have suppressed leptin concentrations compared to age-, weight-, and body fat-matched controls (112).

Studies investigating the relationship between leptin and amenorrhea are difficult to interpret because, although leptin may have a role in regulating reproduction (70), leptin concentrations are tightly regulated by body fat stores (101). Suppressed leptin concentrations in women with amenorrhea are often reflective of decreased body fat (64, 113). For example, when an exercising control group is used to evaluate leptin concentrations in women FHA, leptin concentrations are suppressed in both exercising women with FHA and exercising control women compared to sedentary women (35, 114). De Souza et al. (8, 35) demonstrated that women with exercise-associated FHA have suppressed leptin concentrations similar to the suppressed leptin concentrations observed in ovulatory exercising women i.e., leptin concentrations are more tightly associated with body fat than menstrual status (8, 35). Interestingly, Laughlin and Yen (111) demonstrate that exercising women with FHA have similar fasting leptin concentrations to athletic controls, but that exercising women with FHA have an absent 24h diurnal pattern of leptin, while the athletic controls have a normal diurnal pattern of 24 hour leptin concentrations (Figure 6), indicating that measuring fasting leptin concentrations may not be sensitive enough to discriminate circulating leptin concentrations in lean populations.

While there is indirect evidence in animal and human models to suggest that ghrelin and PYY both participate in regulating reproduction, researchers have provided ample evidence to suggest that leptin is involved in regulating the hypothalamic-
pituitary-ovarian axis in humans (70, 106, 107) (Figure 4). While it is likely that many factors modulate reproduction and signal adequate energy status to the arcuate nucleus, a critical leptin environment is likely a key signal for reproductive function in humans (107). Welt et al. (70) administered human recombinant leptin to eight women with FHA and demonstrated an increase in mean LH pulse frequency.

![Figure 6](image)

Leptin changes during a 24 hour period as percentage changes from the 24 mean leptin concentration in three groups: sedentary women with eumenorrhea (CS), exercising women with eumenorrhea (CA), and exercising women with amenorrhea (AA). Results are expressed as mean ± SEM. Reprinted from Laughlin and Yen J Clin Endocrinol Metab 1997; 82:318-321, with permission.
Additionally, the leptin administration caused an increase in follicular growth, including an increase in the number of dominant which was associated with an ovulatory menstrual cycle in three of the eight participants during the three month intervention. Welt et al. (70), for the first time in humans, demonstrated that leptin is a key signal for reproductive function and that leptin is one of the metabolic hormones and indicators of energy homeostasis that regulates the hypothalamic-pituitary-ovarian axis. While the findings of Welt and colleagues indicate that leptin is an important regulator of reproductive function, the findings also, potentially, have therapeutic implications (70). However, caution should be exercised because while three months of recombinant leptin treatment clearly improved reproductive function in some women with FHA, leptin therapy also caused a decrease in body weight and fat mass in a population who are already typically underweight (70). Increased caloric intake in women with FHA may be a more appropriate method of improving energy status, body weight, body fat and potentially leptin levels to subsequently have an improvement in reproductive status. Increased caloric intake in exercising women with FHA has been demonstrated to reverse amenorrhea (115, 116); however, to date, no studies in humans have investigated what specific metabolic signals up regulate reproduction and reverse amenorrhea in exercising women with FHA during a period of increased caloric intake. Future studies are needed to investigate the role of ghrelin, PYY, and leptin in regulating the hypothalamic-pituitary-ovarian axis, and if these signals can be altered by improving energy status via increased caloric intake.
**Conclusion**

Exercising women with FHA display adaptations consistent with chronic energy deficiency, including alterations in gut peptides and adipocytokines. Ghrelin, PYY, and leptin all have the ability to cross the blood brain barrier and, in the hypothalamus, can modulate appetite and food intake, and are hypothesized to affect the hypothalamic-pituitary-ovarian axis. Elevated ghrelin and PYY concentrations and suppressed leptin concentrations therefore appear to be involved in the etiology of FHA. Future studies are needed to determine if ghrelin, PYY, or leptin are playing a direct role in regulating the hypothalamic-pituitary-ovarian axis, and if these signals can be altered by improving energy status secondary to increasing caloric intake and initiate the reversal of amenorrhea.
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CHAPTER II part 2


INTRODUCTION

Food intake and eating behaviour are controlled by many gastrointestinal hormones that are secreted from the gut region including the stomach and the intestine. These hormones have unique roles in the digestive process, but also interact with the vagus nerve and/or can cross the blood brain barrier and act as signals to the brain to initiate hunger. Gut hormones, such as peptide YY (PYY), control food intake by signalling satiety to the brain, and since PYY can control food intake, this hormone has a pivotal role in eating behaviour and may also play a role in the clinical consequences of over or under eating as observed in clinical models of obesity and anorexia nervosa.

I. General Physiology of PYY

1a. Secretion and Meal Responses. PYY is a gastrointestinal peptide secreted from the endocrine L cells of the intestine in response to food intake (1-4). The two forms of circulating PYY include PYY_{1-36} and PYY_{3-36}. PYY_{3-36} is the major form of circulating PYY after a meal, while PYY_{1-36} is the main form of circulating PYY during a fasted state (5). An enzyme, dipeptidly peptidase IV (DPP-IV) is an important regulator of the expression of PYY_{3-36}; PYY_{1-36} is release from the L cell in the lumen of the intestine and then DPP-IV removes tyrosine-proline (6) from PYY_{1-36} to create the active from of PYY, PYY_{3-36} (7). PYY_{3-36} is highly selective for the Y2 receptor and acts as an agonist for the Y2 receptor (7). PYY, once released peripherally, can cross the blood brain
barrier and act centrally at the level of the arcuate nucleus in the hypothalamus to contribute to eating behaviour. PYY$_{3-36}$ activates the Y2 receptors to inhibit neuropeptide Y (NPY) and agouti related protein (AgRP), as well activate the pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulate (CART), also in the arcuate nucleus, to decrease food intake (1). The ability of PYY$_{3-36}$ to bind to the Y2 receptor indicates the key pivotal role of this peptide in body weight regulation. Hypothalamic Y2 receptors are involved in both food intake and body weight regulation at the level of the hypothalamus (8).
Circulating PYY is an anorexigenic hormone involved in short-term energy homeostasis, i.e., PYY signals satiety following food intake. PYY concentrations rise in response to a caloric load after a meal (2, 4, 9) and stay elevated for several hours (3). PYY starts to rise a few minutes after the calories are ingested and peak PYY concentrations occur 30 mins after caloric intake (10) (Figure 1). After a meal, PYY is elevated, while after short-term fasting (two to three days) total PYY concentrations are suppressed 40-60% below baseline in lean men and women (11). Peak PYY concentrations are achieved in proportion to the amount of calories ingested (2, 10). While PYY is a short term satiety signal involved in the regulation of caloric intake following a caloric load and subsequent meals following a caloric load, PYY is also a signal for long term energy homeostasis, i.e. during conditions such as anorexia nervosa (12-14) and obesity (1, 15, 16).

The rise in plasma PYY concentrations after a meal is also influenced by the macronutrient content of the meal (17). PYY concentrations are released from the intestine in response to specific macronutrients as evidenced by the higher increase in PYY following high vs. normal protein meals (17). Lipids also modulate the release of PYY from the intestine (18, 19). In normal weight subjects, a high-protein (65% protein, 17% fat and 17% carbohydrate) meal elicited the greatest increase in total PYY and PYY3-36 compared to high-fat (17% protein, 66% fat and 17% carbohydrate) and high-carbohydrate (17% protein, 18% fat and 65% carbohydrate) meals, while the high-fat meal still had a greater increase in total PYY and PYY3-36 compared to the high-carbohydrate meal (Figure 2) (17).
Pharmacological administration of PYY3-36 supports a role for PYY in altering eating behaviour in both animal (20) and human models (1, 10, 15, 21, 22). Peripherally administered PYY3-36 has been shown to decrease appetite and 24-hour food intake by 33% in humans (22) (Figure 3). In another study, PYY3-36 infusion in both lean and obese individuals decreased caloric intake by 30% during a buffet meal (1).

![Figure 2](image)

**Figure 2**

Total plasma PYY (PYY3-36 + PYY1-36) concentrations in normal weight individuals following high-protein (PROT), high-fat (FAT), and high-carbohydrate (CHO) meals. The high-protein meal elicited the greatest increase in total PYY compared to high-fat and high-carbohydrate meals, while the high-fat meal still had a greater increase in total PYY compared to the high-carbohydrate meal. Results are expressed as mean ± SEM, n=10. Reprinted from Batterham et al., Cell Metab 2006; 4: 223-233, with permission.

**Ib. Fasting Measures versus Meal Responses.** Baseline PYY concentrations are typically measured following an overnight fast. Two to three days of fasting will suppress total PYY 40-60% below baseline concentrations (11). The meal responses of PYY3-36 and total PYY concentrations have been investigated following the intake of a
caloric load. Both PYY$_{3-36}$ and total PYY concentrations rise following a caloric load (1, 10, 13). Total PYY concentration increased 208% compared to fasting concentrations of total PYY following a large breakfast of approximately 1000 kcal, while PYY$_{3-36}$ concentration increased 125% compared to fasting PYY$_{3-36}$ concentration following the same large breakfast (13). The rise in PYY concentrations compared to fasting is clearly related to the amount of calories ingested; total PYY concentrations were measured following a light lunch of 500 kcal and a large lunch of 1500 kcal and, although total PYY increased significantly compared to fasting concentrations of PYY following the light lunch, the increase in PYY was small compared to the increase in total PYY concentrations following a large lunch of 1500 kcal (10) (Figure 1).
Assays and Measurement Concerns

Total PYY and PYY3-36 concentrations can be measured in plasma. When measuring total PYY, an antibody is used that recognises both PYY1-36 and PYY3-36. For research purposes, scientists are also interested in measuring PYY3-36 because of the peptide’s direct ability to activate the Y2 receptor and influence food intake and eating behaviour. When measuring PYY3-36, an antibody is used that only has cross-reactivity for the active form of PYY, PYY3-36. When measuring PYY3-36, a DPP-IV inhibitor needs to be used to stop DPP-IV from cleaving PYY1-36 into PYY3-36.

Figure 3

A) Caloric intake during a buffet meal following either saline or PYY3-36 infusion. The lines represent individual subject data. B) Caloric intake during the 24 hour periods following either saline or PYY3-36 infusion. ***p<0.0001 vs saline. Results are expressed as mean ± SEM, n=12. C) Appetite scores expressed as percentage change from baseline following either saline or PYY3-36 infusion. *p<0.05 vs saline, **p<0.01 vs saline. Results are expressed as mean ± SEM, n=12. Reprinted from Batterham et al., Nature 2002; 418: 650-654, with permission.
PYY$_{3-36}$. The addition of the DPP-IV inhibitor needs to occur immediately after the blood is drawn for the most accurate results. The effects of long term storage on total PYY and PYY$_{3-36}$ concentrations are unknown; however, if total PYY breaks down during long term storage of samples, then the relative concentrations of PYY$_{1-36}$ and PYY$_{3-36}$ would likely be compromised.

II. Clinical Populations: PYY and Anorexia Nervosa


Anorexia nervosa is related to the cognitive suppression of food intake, i.e., starvation and undernutrition, that leads to a chronic energy deficiency (23). Interestingly, the etiology of exercise-associated functional hypothalamic amenorrhea (FHA) is also related to energy deficiency and in these women and in women with anorexia nervosa the energy deficiency involves a decrease in resting energy expenditure, and suppression of triiodothyronine, insulin-like growth factor-1 and leptin concentrations and elevated ghrelin and cortisol concentrations (24, 25). Evidence is accumulating that fasting and post-prandial PYY concentrations are chronically elevated in populations of energy deficient women, including women with anorexia nervosa and FHA (12-14, 26, 27). Patients with anorexia nervosa, i.e. a population exhibiting chronic energy deficiency, have consistently been observed to exhibit elevated fasting PYY concentrations (12-14). Exercising women with FHA, secondary to an energy deficit, also demonstrate elevations in fasting PYY concentrations (26, 27).

IIb. PYY Meal Responses in Anorexia Nervosa. Investigators have consistently observed that women with anorexia nervosa have an elevated total PYY and PYY$_{3-36}$ in response to a meal (14, 28). Nakahara et al. (14) have shown that PYY$_{3-36}$ concentrations are
elevated at baseline and following intake of a 400kcal standard meal (20% protein, 22% fat and 58% carbohydrate) in women with anorexia nervosa compared to normal weight controls (Figure 4). Since PYY is an anorexigenic hormone, elevated PYY may play a role in reduced food intake in both women with anorexia nervosa and exercising women with FHA, such that circulating elevated PYY may cause a decrease in hunger and total caloric intake and may contribute to the energy deficiency in these populations of women.

IIc. PYY Responses to Weight Gain in Anorexia Nervosa.

Although PYY concentrations are elevated in women with anorexia nervosa, weight gain in women with anorexia nervosa after treatment for the disease causes decreases fasting and postprandial PYY concentrations compared to before treatment, but this weight gain in women with anorexia nervosa following treatment does not normalize fasting or postprandial PYY concentrations compared to healthy controls (14) (Figure 4).
Notably, elevated PYY concentrations have been hypothesized to be more than just a marker of energy deficiency but rather may play an important role in modulating eating behaviour in women with anorexia nervosa. Misra et al (12), who observed elevated PYY concentrations in a population of adolescent girls with anorexia nervosa, suggested that elevated PYY may be involved in the pathogenesis of anorexia nervosa by way of reducing food intake. PYY is an anorexigenic hormone and elevated PYY reduces food intake in lean individuals (1). Exercising women with FHA are also an undernourished clinical population in an energy deficit. Similar to women with anorexia nervosa, exercising women with FHA demonstrate elevations in fasting PYY concentrations (26, 27) (Figure 5).

This finding suggests that elevated PYY may cause a decrease in relative caloric intake and may compound the psychological struggle in women attempting to recover from anorexia nervosa and consume food for the purpose of weight gain. Thus, in general, elevated PYY in women with anorexia nervosa and exercising women with FHA may contribute to energy deficiency by way of causing a compensatory decrease in food
intake and may represent a component of the underlying mechanism involved in suppressed food intake in these women.

**IlD. PYY and Eating Behaviours in Anorexia Nervosa and FHA.** PYY may play a role in modulating eating behaviours and suppressing food intake by interfacing with other gastrointerstinal peptides at the level of the hypothalamus. Interestingly, ghrelin concentrations are also elevated in women with anorexia nervosa (14, 29-32) and in exercising women with FHA (26, 33, 34). Elevated ghrelin concentrations in energy deficient populations, such as women with anorexia nervosa or FHA are part the body’s attempt to increase food intake and an increase in food intake would then, presumably, help to reverse energy deficiency. Ghrelin is an orexigenic hormone and infusions during a buffet meal are associated with an increase in food intake (35). Elevated PYY in the presence of elevated ghrelin may cause a relative ghrelin resistance in women with anorexia nervosa (12), i.e., in spite of chronically elevated ghrelin these is no compensatory increase in food intake. Ghrelin resistance may represent the underlying mechanism explaining why undernourished women with elevated ghrelin concentrations are not increasing food intake. Interestingly, PYY has been shown to inhibit ghrelin neurons (36) and may represent a physiological mechanism explaining ghrelin resistance. Women with elevated PYY concentrations such as women with anorexia nervosa consistently express behaviours including restricting food intake even in the presence of elevated physiological signals, like ghrelin, to increase food intake. Elevated PYY may be disrupting food intake signals in the uncoupled relationship between elevated ghrelin and food intake in women with anorexia nervosa.
Although there is no direct evidence to date to demonstrates that PYY is involved in the etiology of anorexia or exercise associated FHA, Scheid et al. (26) found an association between fasting PYY concentrations and drive for thinness. High drive for thinness includes disordered eating behaviours that encompass a fear of gaining weight and a preoccupation with body weight and body shape (37, 38). High drive for thinness has been physiologically linked to an energy deficiency in exercising women (39), and it is therefore not surprising that PYY is also associated with a high drive for thinness. However, if PYY is indeed a physiological signal that is suppressing food intake, this signal may be compounding disordered eating behaviours in women with anorexia and exercising women with FHA.

III. Clinical Populations: PYY and Obesity

IIIa. Fasting PYY in Obesity. PYY concentrations are suppressed in some (1, 15, 40, 41), but not all studies of (13) obese patients, a population exhibiting a long-term positive energy imbalance. Fasting PYY is negatively correlated with body mass index (BMI) demonstrating that individuals with suppressed fasting PYY concentrations have elevated BMIs and may be overweight/obese (42). Suppressed PYY in obesity may play a role in increased food intake in this population given PYY’s important role in promoting satiety. Indeed, Batterham et al. (1) suggest that suppressed PYY concentrations may be involved in the pathogenesis of obesity, suggesting that obese individuals may be predisposed to low circulating PYY concentrations, leading to an increase in food intake and weight gain.

IIIb. PYY Meal Responses in Obesity. Obese patients demonstrate an attenuated total PYY response to a caloric load (1, 15, 16). Total circulating PYY concentrations are
consistently suppressed in obese individuals compared to lean individuals prior to, during and following a buffet lunch (Figure 6). The attenuated PYY response to a caloric load during obesity leads to an increase in food intake and is one of the factors that make weight control difficult in obese populations (1). Le Roux et al. (15) investigated the meal response in both obese and lean subjects and found that the obese group had a consistently attenuated PYY response during a 1000kcal liquid meal and also during a liquid meal that totalled almost 3000 kcal. They found that the obese group needed to consume almost double the caloric load to achieve the same PYY concentration as the lean group, potentially demonstrating that an attenuated PYY response to a caloric load may lead to overeating and weight gain in obese individuals.

Figure 6

Total circulating PYY concentrations in an obese group (triangles) compared to a lean group (circles) during infusion of saline and after a buffet lunch. Total circulating PYY concentrations are consistently suppressed in the obese group compared to the lean group during infusion of saline and after a buffet lunch. Results are expressed as mean ± SEM, n=24. Reprinted from Batterham et al. The New England journal of medicine 2003; 349:941-948, with permission.
Illc. *PYY Responses to Weight Loss in Obesity.* Prospective studies that utilize weight loss interventions to create an energy deficit offer valuable insights into the role of PYY in long term energy homeostasis, but results have been inconsistent and thus difficult to interpret. Consistent with the concept that fasting PYY concentrations are elevated in situations following long-term energy deficiency, fasting PYY concentrations are elevated in obese children after weight loss (40) and adolescents (43) after exercise training. Roth et al. (40) investigated the effects of a one year outpatient program that included exercise, nutrition education and individual counselling on fasting total PYY concentrations in obese children. Roth and colleagues demonstrated that in the quartile of children with the greatest BMI reduction the greatest percentage increase in total PYY concentrations was observed. Jones et al. (43) investigated the effects of a 32 week exercise training program on PYY concentrations in obese adolescents and demonstrated that total PYY concentrations were increased following the training compared to baseline PYY concentrations, indicating that exercise training is the absence of weight loss may affect circulating PYY concentrations, however the mechanism behind this increase in PYY is unknown.

Unfortunately, the weight loss literature regarding PYY is very inconsistent and while in obese children who lose weight have an increase in circulating PYY concentrations (40), other studies in obese adults have reported that PYY concentrations decrease after weight loss (13, 44), indicating that PYY concentrations may be regulated different in adults and children. The inconsistencies in the literature regarding PYY
concentrations following weight loss may also be due to the notion that some obese individuals may be predisposed to low circulating PYY concentrations (1), meaning that they already have low PYY concentrations and that these low PYY concentrations may respond differently to weight loss.

An additional complication in the weight loss literature is that fasting PYY concentrations may not be sensitive to smaller changes in weight, particularly in normal weight populations. For example, in our laboratory (Leidy et al., 2004; Scheid et al, in review) we implemented a well controlled and intensive three month exercise and diet intervention that produced weight loss (2-4kg) in normal weight women 18-30 years old. The weight loss produced was associated with changes in energy balance evident by elevated ghrelin concentrations and suppressed resting metabolic rate (45). However, we (Scheid et al, in review) observed that total PYY concentrations were not altered by diet and exercise-induced weight loss in these women. Future research will need to investigate the relationship of weight loss and initial PYY concentrations and if changes in PYY concentrations following weight loss impact eating behaviour and weight maintenance.

**IIIId. PYY Infusion Studies in Obesity.** Since PYY is a peripherally circulating hormone that signals satiety, pharmacological administration of PYY has reportedly been referred to as having a potential application as a weight loss drug. Pharmacological administration of PYY$_{3-36}$ has been shown to decrease food intake in both animal (20) and human models (1, 10, 15, 21, 22). Peripherally administered PYY$_{3-36}$ has been shown to decrease appetite and 24-hour food intake by 33% in humans (22) (Figure 3). Batterham et al. (1) investigated the effects of PYY$_{3-36}$ infusion in both lean and obese
individuals and found that PYY infusion decreased caloric intake by approximately 30% in both the lean and obese individuals during a buffet meal. The authors suggest that these data indicate that obese subjects may in fact not be PYY resistant, but rather that they may simply be PYY deficient. These studies also suggest that the administration of PYY$_{3-36}$ may be a future weight loss aid by potentially decreasing caloric intake during meals (1).

**IIIe. PYY and Eating Behaviours in Obesity.** Suppressed PYY in obesity may play a role in increased food intake in this population given PYY’s important role in promoting satiety. Indeed, fasting total PYY concentrations are suppressed in obese patients, and of greater concern, obese patients demonstrate an attenuated total PYY response to a caloric load (1, 15, 16). Since obese individuals have an attenuated response during food intake, obese individuals likely do not experience the same satiety signals as lean individuals. Obese individuals may need to consume almost double the caloric load to achieve the same PYY concentration associated satiety as the lean group (15). Suppressed PYY may therefore represent be a physiological factor that leads to increased food intake in obese individuals and may make weight loss difficult for these people.

Macronutrient meal composition can also affect PYY$_{3-36}$ secretion in obese populations and lead to alterations in food intake. The rise in plasma PYY$_{3-36}$ concentrations after a meal is influenced by the macronutrient content of the meal; however the PYY$_{3-36}$ response to macronutrients differs between lean and obese individuals. A high-protein meal is associated with the greatest increase in total PYY and PYY$_{3-36}$ compared to high-fat and high-carbohydrate meals in both lean and obese individuals (17). On the other hand, a high-fat meal is associated with a greater increase
in total PYY and PYY\textsubscript{3-36} compared to a high-carbohydrate meal only in the lean group, suggesting that a high protein diet would be beneficial for obese individuals, potentially increase satiety, and may assist with weight control or weight loss (17). Misra et al. (46) fed obese and lean adolescent girls a high fat, high carbohydrate, and high protein meals and measured the % change in PYY\textsubscript{3-36} and active ghrelin concentrations. The investigators reported suppressed PYY\textsubscript{3-36} concentrations in the obese girls after the high fat meal, but no difference between the lean girls after the high carbohydrate and high protein meals. Interestingly, food intake following the high fat meal was increased in the obese girls, suggesting that suppressed PYY\textsubscript{3-36} concentrations in obese girls’ influences subsequent food intake. The findings of Misra and colleagues (46) further indicated that a high carbohydrate meal caused an increase in ghrelin concentrations in obese girls also leading to an increase in food intake and that high protein meals did not alter PYY\textsubscript{3-36} secretion or ghrelin secretion in the obese girls and did not increase food intake compared to the controls. These findings suggest that macronutrient composition of meals such as high protein meals could increase satiety in obese populations and contribute to weight control.

**IIIf. PYY and Genetic Obesity.** While elevated PYY has been suggested to play a role in the pathogenesis of anorexia nervosa, Batterham et al. (1) suggest that suppressed PYY concentrations may be involved in the pathogenesis of obesity. Boey et al (47) investigated the relationship between suppressed total PYY and a genetic predisposition towards obesity and diabetes and suggested that suppressed fasting PYY concentrations may indeed indicate a genetic predisposition that is associated with obesity and type 2 diabetes. Suppressed fasting PYY concentrations are also observed in females with first-
degree relatives who have type 2 diabetes (47) further supporting the idea that obesity may be linked to a PYY deficiency.

IV. Applications to other areas of health and disease

IVA. PYY and Reproduction Suppression. Women with anorexia and FHA both experience suppressed reproductive function and elevated PYY concentrations (12, 26, 27). Many metabolic and gastrointestinal hormones are hypothesized to cross the blood brain barrier and interact with NPY in the arcuate nucleus and POMC in the paraventricular nucleus to effect the hypothalamic-pituitary-ovarian axis as well as appetite (48). There is evidence that PYY may be directly participating in the down regulation of reproductive function. Animal models in Syrian hamsters have shown that infusions of PYY inhibit estrous (49). Human studies have shown that the human placenta contains PYY (50). There is a plausible role for PYY to be one of the metabolic and gastrointestinal hormones involved in the suppression of reproductive function. More research is needed to explore to extent of PYY’s role in modulating female reproductive function.

IVB. PYY and Suppressed Bone Health. Women with anorexia and FHA both demonstrate a decrease in bone mineral density (37, 51-54). Women with amenorrhea experience increased bone resorption related to estrogen deficiency and a decrease in bone formation related to an energy deficiency (52). Elevated PYY is associated with suppressed bone mineral density (51, 55) and suppressed markers of bone formation (12). An elevation in PYY associated with an energy deficiency may be directly participating in a central mechanism to suppress bone formation and decrease bone mineral density in energy deficient populations. Utz et al. (51) investigated women with anorexia nervosa
and found a negative correlation between mean PYY concentrations (samples over 12 hours) and bone mineral density. A plausible mechanism explaining the relationship between elevated PYY concentrations and a decrease in bone mineral density comes from the animal literature. Y2 deficient mice have shown that hypothalamic Y2 receptors regulate bone formation and suppression in Y2 receptor activation increases bone volume (56). PYY3-36 activates the hypothalamic Y2 receptor and down regulates NPY release and through this central mechanisms related to a down regulation of NPY, PYY appears to be decreasing bone mineral density (51).

**Conclusions**

PYY is a satiety hormone involved in short-term food intake highlighted by the rise in PYY3-36 following a meal, as well as pharmacological administration of PYY3-36 causing a decrease in caloric intake. PYY concentrations are altered in conditions reflecting long-term disturbances in energy homeostasis such as anorexia nervosa and obesity. Elevated PYY may be involved the pathogenesis of anorexia nervosa and elevated PYY concentrations may play a role in preventing compensatory increases in food intake, while suppressed PYY concentrations may be involved in the pathogenesis of obesity may play a role in preventing weight control.
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CHAPTER III


ABSTRACT

Purpose: The aim of this study was to examine changes in fasting total PYY and ghrelin in non-obese premenopausal women following an exercise and diet program with and without weight loss. Methods: Body composition, energy balance parameters, ghrelin, and PYY were measured before and after a three month intervention in non-exercising controls (n=7), and exercising women who either remained weight stable (n=5) or lost weight (n=10). At baseline subjects were 20.6±2.2 years, weighed 58.0±4.8 kg, and were 27.2±4.9% body fat. Supervised exercise training occurred five times a week for up to 90 min at 70-80% of maximum heart rate. Subjects were fed a controlled diet. Results: Body weight (-3.2±0.8 kg) and fat mass (-2.6±0.7 kg) decreased significantly in the weight-loss exercise group. Neither fasting ghrelin nor PYY changed in response to exercise training in the absence of weight loss, and PYY did not change with exercise and weight loss. Fasting ghrelin did reveal a significant time by experimental group interaction (p=0.025). The change in ghrelin was inversely correlated with the change in body weight, BMI, fat-free mass and energy availability. Conclusions: Neither fasting ghrelin nor fasting PYY appear to play a role in the adaptive changes associated with exercise training when exercise occurs in the absence of weight loss. Fasting ghrelin concentrations increase when body weight is lost and may respond to even smaller
changes in energy availability. However, fasting PYY does not appear to play a key role in the regulation of energy balance during diet and exercise associated weight loss.

INTRODUCTION

Ghrelin and Peptide YY (PYY) are both gastrointestinal peptides; PYY is secreted from the endocrine L cells of the ileum of the intestine (1, 2), while ghrelin is secreted by distinct endocrine cells of the stomach called X/A-like cells or ghrelin cells (3). Both ghrelin and PYY appear to be involved with appetite-regulation and energy homeostasis. Specifically, ghrelin concentrations respond rapidly to a negative energy balance, induced by either small meals or exercise (4). Fasting ghrelin concentrations are elevated following weight loss due to a low calorie diet (5, 6) and combined diet and exercise (7, 8), demonstrating a role for ghrelin in the regulation of long-term energy homeostasis. Although PYY appears to play an important role in short-term energy homeostasis (9), signaling satiety (10) and potential meal termination (11), the evidence supporting PYY in a long-term role in energy balance regulation is conflicting. Fasting total PYY concentrations are increased in obese children following exercise training and weight loss (12). In contrast, Pfluger et al. (13) and Lien et al. (14) demonstrated a decrease in fasting total PYY concentrations in obese patients after weight loss. More research is necessary to understand the physiological relevance of alterations in fasting total PYY in both clinical and healthy populations.

As ghrelin and PYY appear to have opposing actions, the relationship between alterations in PYY and ghrelin may play a role in weight loss and or weight maintenance in healthy populations. Riediger et al. (15) demonstrated that PYY can inhibit ghrelin neurons, indicating that PYY concentrations may be important when evaluating changes
in ghrelin concentrations. To date, no studies have evaluated both fasting total PYY and ghrelin concentrations before and after weight loss in healthy non-obese women. Thus the roles of ghrelin and PYY in the maintenance of long term energy balance in the absence of pathophysiology such as obesity or anorexia are unknown. The exploration of potential interactions between PYY and ghrelin may help elucidate the complex physiology of energy homeostasis. Prospective weight loss studies offer valuable insights into the roles of and possible interactions between both PYY and ghrelin in body weight regulation in non-pathological conditions.

The term energy availability generally reflects the energy available for physiological functions over and above maintenance energy requirements. Energy availability has been specifically defined as the difference between daily energy intake and the energy expended during purposeful exercise adjusted for lean body mass (16, 17). Loucks et al. (16, 17) and others (4) have shown that energy availability has been strongly related to changes in metabolic hormones such as triiodothyronine (16), leptin (17) and ghrelin (4) in exercising women. Borer and colleagues (4) manipulated caloric intake and exercise energy expenditure during a one day intervention and demonstrated that ghrelin concentrations respond rapidly to short-term changes in energy availability. To date, no studies have explored how manipulating energy availability for three months impacts circulating ghrelin and PYY concentrations.

The purpose of this study was threefold: 1) to examine fasting total PYY and total ghrelin concentrations in non-obese premenopausal women before and after a 3 month exercise and diet program where energy availability is reduced leading to weight loss, 2) to examine whether exercise training per se, in the absence of weight loss, leads to
changes in circulating total PYY and total ghrelin, and 3) to examine whether changes in these peptides occur in relation to each other. We hypothesized that women in an exercise and diet program that results in weight loss will experience a decrease in fasting total PYY and an increase in fasting total ghrelin concentrations consistent with a possible role of both total ghrelin and total PYY in long-term regulation of energy balance by acting in a compensatory manner to increase food intake and thus restore energy balance. We also hypothesized that increasing energy intake to match the higher energy expenditure and maintain body weight will not have a significant effect on fasting total ghrelin or PYY. Lastly, we hypothesized that changes in ghrelin and PYY would be significantly related to each other.

SUBJECTS AND METHODS

Experimental design overview

This study was part of a larger prospective study designed to assess changes in reproductive function in response to a controlled exercise and feeding intervention. Inclusion in the larger study was based on the following: 1) no history of serious medical conditions; 2) no current evidence of disordered eating or history of an eating disorder 3) age, 18–30 years; 4) weight 45–75 kg; 5) body fat, 15–35%; 6) BMI, 18–25 kg·m²; 7) nonsmoking; 8) no medication use that would alter metabolic or reproductive hormone concentrations; 9) no significant weight loss/gain ±2.3kg (±5lbs in the last year); 10) less than 1 hour of purposeful aerobic exercise per week, 11) not taking hormonal contraceptives for the past 6 months, 12) documentation of at least two ovulatory menstrual cycles, and 13) suitable candidate for a controlled feeding and exercise study.
Each subject was informed of the purpose, procedures, and potential risks of participation in the study before signing a written informed consent approved by the Penn State University Biomedical Institutional Review Board. Both total ghrelin and total PYY were measured in blood samples from a subset of subjects from the larger study who completed the study and either exercised but remained weight stable (+1.5kg to -1.25kg), exercised and lost weight (at least -1.5kg) or served as non-exercising controls and remained weight stable (+1.35kg to -1.25kg).

**Screening**

To determine inclusion in the study, subjects completed surveys of medical history, physical activity, eating attitudes (18, 19), underwent a physical examination by a clinician at the General Clinical Research Center (GCRC), had a screening blood draw to rule out endocrine or metabolic disease (Quest Diagnostics, Madison, NJ) and had an interview that was supervised by a clinical psychologist to rule out disordered eating.

**Subject Groupings**

Following screening all subjects were monitored for a baseline period (one menstrual cycle) followed by three menstrual cycles (~3 months) during which the ~3 month controlled diet and supervised exercise intervention occurred. Before the baseline period, subjects were randomly assigned to one of the study groups; a control group who performed no exercise and were provided enough calories to maintain initial body weight, an energy balance group that exercised at a high level and were provided extra calories to match those expended through exercise, and three exercising groups that were fed a reduced calorie diet. Energy intake was quantified daily, and exercise energy
expenditure was quantified during each workout. We performed an intent to treat analysis to test if women in an exercise and diet program would experience a decrease in fasting total PYY and an increase in fasting total ghrelin concentrations. PYY and ghrelin were analyzed using an ANOVA with repeated measures. We found no effect of the intervention on PYY or ghrelin, even though we successfully increased exercise expenditure (p<0.001) in both exercise groups compared to the control group, decreased caloric intake (p<0.001) in exercise and caloric restriction group compared to the other groups and increased caloric intake (p<0.012) in the exercising control group compared to the other groups. Although our intervention successfully reduced dietary intake and increased exercise we did not observe the degree of weight loss (exercise and caloric restriction group) or weight maintenance (exercising control group) that would have likely been achieved if subject compliance was optimal. Because we were interested in the impact of weight loss achieved through exercise and caloric restriction, and the impact of exercise alone, we performed a secondary analysis based on weight loss. For the current study, subjects were regrouped based solely on their exercise and weight loss status after completion of the intervention into the following three groups: 1) control group (no exercise, weight maintenance diet), 2) weight-stable exercisers (exercised, weight maintenance diet, and 3) weight-loss exercisers (exercised and lost a significant amount of weight). To determine the magnitude of weight change deemed to represent significant weight loss, the normal variability in body weight in this group of subjects was estimated by examining the maximum weight change observed during the study in the control group, which was ±1.5 kg. Thus, a change in body weight of 1.5 kg or more became the minimum criterion for inclusion into the weight-loss exercising group,
whereas those with weight gain/loss less than ±1.5 kg were included in the weight-stable exercising group.

**Dietary intake during the intervention**

The study was designed to provide all the food subjects were to eat over the entire three month intervention. All food for the study was prepared and weighed to the nearest gram to achieve the desired calorie level by GCRC metabolic kitchen staff. Energy content of prepared food items were verified with bomb calorimetry. Subjects were required to eat two of three meals per day during the week at the GCRC dining room. Meals during weekends, dinners, and a daily snack were packed out. The diet was comprised of 55% carbohydrates, 30% fat, and 15% protein. The calorie level required to maintain weight for each subject was calculated based on previously published methods (20). To meet the target level of caloric intake during the intervention, intake was either increased or reduced from the weight maintenance level depending on the experimental group. Subjects were instructed to eat all, and only, the food provided to them by the study. Any uneaten food was reweighed and recorded for later subtraction from the prescribed total intake using previously published methods (20). Daily and weekly averages of 24-h calorie intake were calculated throughout the study. During the intervention, body weight and 24-h energy expenditure were repeatedly monitored and minor adjustments in caloric intake levels and exercise energy expenditure were made.

**Exercise training protocol during the intervention**

All exercise training took place in the laboratory and was supervised by trainers who had experience in fitness assessment and personal training. Both the weight-loss and
weight-stable exercising groups performed aerobic exercise five times per week at 70–
80% of maximum heart rate as determined from tests of maximal aerobic capacity
(VO₂max). Exercise duration ranged from 20 to 90 minutes as training progressed.
Caloric expenditure during exercise sessions was 550±62 kcal in weight stable exercisers
and 470±122 kcal in the weight loss exercisers (p=0.117). Exercise was not prescribed on
“off” days, and subjects were asked to maintain their non-purposeful physical activity at
levels equivalent to Baseline throughout the study. The duration of exercise was
progressively increased throughout the intervention.

**Energy Expenditure**

Activity energy expenditure was monitored every other week for 7 days using
accelerometers so that unusual deviations from typical patterns of physical activity could
be monitored. The total amount of calories expended during each exercise session was
measured using the OwnCal feature on the Polar S610 heart rate monitor (Polar Electro
Oy, Kempele, Finland). This feature uses body weight, height, age, gender, VO₂max,
individual maximum HR, individual HR in a sitting position, and HR during exercise to
derive kilocalories from energy expenditure. The OwnCal feature has been validated for
the use in calculating exercise energy expenditure from heart rate (21-23). This feature
uses body weight, height, age, gender, VO₂max, individual maximum HR, individual HR
in a sitting position, and HR during exercise to derive kcals from energy expenditure.
HR monitoring underestimates total energy expenditure by a mean value of 1.2 (SD 6.2)
% (range -11.4 to 10.6%) (p>0.05) compared to indirect whole-body calorimetry (24).
Modes of aerobic activity for both exercise groups included treadmill walking and
running, stationary cycling, stair stepping, and use of an elliptical ergometer.
Body composition, resting metabolic rate, and cardiorespiratory fitness assessment

All of the following measurements were completed during the pre-, and post-intervention periods in all subjects. Body fat percentage, fat mass and fat-free mass were determined by hydrostatic weighing after correcting for residual lung volume (20, 25). Body weight was measured using a digital scale to the nearest 0.01kg (Seca scale, Hamburg, Germany) on the same day that body composition was determined and twice per week throughout the intervention. Resting metabolic rate (RMR) was measured during baseline and post-study between 0600-1000 h after an overnight fast using indirect calorimetry (20). Measurement of maximal aerobic capacity (VO$_2$max) using a treadmill and open circuit spirometry was performed during baseline and post-study (20).

Energy Availability

Body weight changes can be deceiving, since physiological adjustments to an energy deficiency, e.g., alterations in circulating ghrelin, have been reported in the absence of weight loss (26). Therefore, in addition to changes in body weight we also measured changes in energy availability. Energy availability takes into consideration daily fluctuations of daily energy intake and daily fluctuations in exercise energy expenditure, correcting for fat free mass. The latter definition of energy availability does not account for energy expenditure other then exercise energy expenditure. Short term manipulations of energy availability, i.e. carefully controlling energy intake and exercise expenditure, have consistently demonstrated energy availability to be related to changes in metabolic hormones such as triiodothyronine (16), leptin (17) and ghrelin (4), and reproductive hormones such as luteinizing hormone (27). Consistent with the treatment of
this variable in the literature (16, 17, 27), we operationally defined energy availability as:
\[
\frac{\text{Energy intake (kcal)} - \text{exercise energy expenditure (kcal)}}{\text{Fat-free mass (kg)}}.
\]

**Blood Sampling**

Fasting morning blood samples were collected during the early follicular phase (menstrual cycle days 1-7) of the baseline, beginning of the third intervention cycle, and during the post-intervention menstrual cycle between 0700 -1000 hr at the GCRC after subjects lay supine for at least 15 min. Samples were allowed to clot for 1 hour before they were processed. Subjects did not exercise for at least 12 hours prior to blood sampling. Serum aliquots were then stored at -80°C and samples remained frozen prior to assay.

**Biochemical Analyses**

Total PYY was measured on only pre and post-intervention time points using an RIA for total PYY (Linco Research, St. Charles, MO). The total PYY assay recognizes both PYY1-36 and PYY3-36 and does not require the addition of inhibitors. The intra-assay and inter-assay coefficients of variation were 5.3 and 7.0%, respectively. The sensitivity of the assay was 10 pg/ml. Total ghrelin was measured during pre, mid, and post-intervention time points using an RIA for total ghrelin (Linco Research, St. Charles, MO). The intra-assay and inter-assay coefficients of variation were 2.0 and 15.7%, respectively. The sensitivity of the assay was 93 pg/ml. All samples from a given subject were analyzed in duplicate and in the same assay from unthawed serum aliquots.
Statistical analyses

Data screening was conducted prior to analysis, involved outlier detection, and examination of variable distributions within each of the groups for normality. Since all of the distributions were normal, parametric analyses were utilized. An ANOVA with repeated measures was performed on PYY at pre- and post intervention and on ghrelin at pre-, mid-, and post intervention. When main effects were detected, post hoc analyses were performed using *t* tests employing the Bonferroni correction factor. Baseline measurements as well as change scores calculated from pre- and post study time points for other key variables, were examined using a one-way ANOVA. When main effects were detected, post hoc analyses were performed using the least-significant difference procedures. Analysis of covariance was employed when there were baseline group differences. Pearson product correlation coefficient analyses were used to examine the relationship between changes in total PYY, total ghrelin and other variables of interest. In all analyses, P<0.05 was considered statistically significant. Statistical analyses were performed using SPSS software (version 16.0; SPSS Inc., Chicago, IL). Data are reported as mean ± sd. A power calculation was performed to determine sample sizes required to detect differences. For total PYY, sample size was based on the detection of a meaningful difference of 13.0 pg/ml and an SD of 7.55 pg/ml after a weight-loss program, based on previously published reports (13). In order to achieve 80% power for the total PYY analysis using a 0.05 level of significance, a sample size of 5 participants per group (total n= 15) was required. For ghrelin, sample size was based on the detection of a meaningful difference of 574 pg/ml and an SD of 306 pg/ml after a weight-loss program, based on previously published reports (20). In order to achieve 80% power for
the total PYY analysis using a 0.05 level of significance, a sample size of 5 participants per group (total n= 15) was also required.

RESULTS

Baseline

Baseline descriptive data for subjects in control, weight-stable exercise, and weight-loss exercise groups are shown in Table 1. No significant differences among groups existed in initial body weight or fat-free mass. However, there was a main effect of initial body fat, fat mass, RMR, fitness level and calorie intake. Initial body fat, fat mass and RMR were significantly higher in the weight loss exercisers compared with the controls and weight-stable exercisers. Baseline calorie intake was lower in the control group compared with both exercising groups. Baseline fitness was higher in the weight-stable exercisers compared to the controls. There were no differences in baseline energy availability between the groups.
Table 1. Baseline characteristics before a 3-month diet and exercise program in 22 non-obese young women.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=7)</th>
<th>Weight-stable exercisers (n=5)</th>
<th>Weight-loss exercisers (n=10)</th>
<th>Main Effect of Group p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>21.3±3.5</td>
<td>20.2±1.1</td>
<td>20.3±1.6</td>
<td>0.631</td>
</tr>
<tr>
<td>Body Weight (kg)</td>
<td>55.5±4.1</td>
<td>56.6±3.8</td>
<td>60.4±4.9</td>
<td>0.089</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>25.1±4.4</td>
<td>23.5±4.1</td>
<td>30.5±3.5</td>
<td>0.006</td>
</tr>
<tr>
<td>Fat-Free Mass (kg)</td>
<td>41.5±2.4</td>
<td>43.2±2.8</td>
<td>41.9±3.1</td>
<td>0.574</td>
</tr>
<tr>
<td>Fat Mass (kg)</td>
<td>14.0±3.2</td>
<td>13.3±2.9</td>
<td>18.5±3.0</td>
<td>0.006</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>20.9±1.4</td>
<td>20.0±2.3</td>
<td>22.2±1.9</td>
<td>0.123</td>
</tr>
<tr>
<td>RMR (kcal/day)</td>
<td>1195±144</td>
<td>1195±159</td>
<td>1368±72</td>
<td>0.014</td>
</tr>
<tr>
<td>VO_{2max} (ml/kg min)</td>
<td>31.6±3.3</td>
<td>38.6±3.3</td>
<td>36.1±4.5</td>
<td>0.034</td>
</tr>
<tr>
<td>Intake (kcal/24 h)</td>
<td>1743±310</td>
<td>2040±89</td>
<td>2010±173</td>
<td>0.036</td>
</tr>
<tr>
<td>Energy Availability (kcal/kg•FFM)</td>
<td>42.0±7.4</td>
<td>43.9±3.8</td>
<td>45.1±4.9</td>
<td>0.574</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD. Significance of P < 0.05 using least-significant difference post hoc analyses. BMI, Body Mass Index; RMR, Resting metabolic rate; FFM, Fat-free Mass.

a Weight-loss exercisers vs. weight-stable exercisers and controls.

b Weight-stable exercisers vs. controls.

c Weight-loss exercisers and weight-stable exercisers vs. controls.
Effects of the Intervention on body composition, fitness, and metabolic parameters

Changes in body composition, fitness, and energy availability are shown in Table 2. As expected based on group assignment, significant main effects for changes in body weight and body composition where observed in the experimental group. These significant main effects were still present when baseline differences in body weight and body composition were controlled for. Body weight and fat mass decreased in the weight-loss exercise group compared to both the weight stable exercisers and the controls. Body weight changes ranged from -2.10 kg to -4.33 kg in the weight loss group, +1.5 kg to -1.25 kg in the weight-stable exercising group, and +1.35 kg to -1.25 kg in the control group. There was a trend for main effect on the change in VO2max. The exercising groups showed a 20.6±8.9% increase in VO2max while the controls showed an 11.8 ± 9.2%. When controlling for baseline group differences in VO2max there was a significant group effect of change in VO2max (p=0.003) in that the weight-stable exercise, and weight-loss exercise groups experienced a significantly greater change in fitness than the control group. Additionally, there was also a trend for a main effect of group on the change in energy availability (Table 2).
Table 2. Changes in fitness, body composition, and energy balance parameters following 3-month diet and exercise program.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=7)</th>
<th>Weight-stable exercisers (n=5)</th>
<th>Weight-loss exercisers (n=10)</th>
<th>Main Effect of Group p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ VO₂max (ml/kg min)</td>
<td>3.7±2.9</td>
<td>8.7±2.2</td>
<td>7.1±3.8</td>
<td>0.070</td>
</tr>
<tr>
<td>Δ Body Weight (kg)</td>
<td>0.2±0.8</td>
<td>0.5±1.2</td>
<td>-3.2±0.8 a</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Δ Body Fat (%)</td>
<td>0.3±1.6</td>
<td>0.3±2.1</td>
<td>-2.9±1.2 a</td>
<td>0.001</td>
</tr>
<tr>
<td>Δ Fat-Free Mass (kg)</td>
<td>-0.1±1.0</td>
<td>0.3±1.1</td>
<td>-0.6±0.7</td>
<td>0.198</td>
</tr>
<tr>
<td>Δ Fat Mass (kg)</td>
<td>0.3±1.1</td>
<td>0.3±1.4</td>
<td>-2.6±0.7 a</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Δ BMI (kg/m²)</td>
<td>0.1±0.3</td>
<td>0.2±0.4</td>
<td>-1.2±0.3 a</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Δ RMR (kcal/day)</td>
<td>115±158</td>
<td>158±158</td>
<td>-43±86 a</td>
<td>0.020</td>
</tr>
<tr>
<td>Δ Exercise (kcal/24 h)</td>
<td>0±0</td>
<td>345±48 b</td>
<td>300±86 b</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Δ Intake (kcal/24 h)</td>
<td>68±89</td>
<td>89±371</td>
<td>-87±345</td>
<td>0.485</td>
</tr>
<tr>
<td>Δ Energy Availability (kcal/kg•FFM)</td>
<td>0.6±0.8</td>
<td>-4.0±6.3</td>
<td>-6.6±6.7</td>
<td>0.073</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD. Significance of P < 0.05 using least-significant difference post hoc analyses. BMI, Body Mass Index; RMR, Resting metabolic rate; FFM, Fat-free Mass.

a Weight-loss exercisers vs. weight-stable exercisers and controls.

b Weight-loss exercisers and weight-stable exercisers vs. controls.
Prescribed calorie intake and actual calorie intake varied less than 35 calories per week during the intervention, and no differences in this parameter existed among groups. Final macronutrient intake was in accordance with that prescribed i.e., of 55% carbohydrates, 30% fat, and 15% protein with no differences between groups. With respect to compliance to the exercise training protocol, 14 of the 15 exercisers (93%) consistently reached their prescribed exercise calorie level per week and target exercise intensity level (70–80% of maximal heart rate).

**PYY and Ghrelin**

No differences (p>0.05) were observed in fasting total PYY concentrations among groups before the intervention. In response to the intervention, when pre- and post-time points for PYY were analyzed using an ANOVA with repeated measures, no significant effects were observed (Figure 1). No significant main effects were observed in PYY when controlling for baseline differences in body composition or fitness levels. No differences (p>0.05) were observed in fasting total ghrelin concentrations among groups before the intervention. However, in response to the intervention pre-, mid- and post-time points did reveal a significant (p=0.025) time by experimental group interaction for fasting total ghrelin concentrations using an ANOVA with repeated measures (Figure 2). The main effect of time by experimental group interaction was still present when controlling for baseline differences in body composition (body fat), food intake or fitness level. A post hoc analysis using t tests employing the Bonferroni correction factor reveal that the weight loss group has a significant (p=0.001) increase in fasting ghrelin concentrations from pre- to post.
Figure 1

Pre- and post-time points for fasting PYY were analyzed using an ANOVA with repeated measures, no significant main effects were observed (n=22). Values are expressed as mean ± SD.

Pearson correlations between change in PYY, change in ghrelin and changes in body composition and changes in energy availability are presented in Table 3. There were no correlations between change in total PYY and change in body weight, BMI, fat mass, fat-free mass, or energy availability. However, the change in ghrelin was associated with the change in body weight, BMI, fat-free mass, and energy availability. The relationship between the changes in PYY and ghrelin and the change in body weight is shown in Figure 3. Additionally, there was no correlation between the change in
ghrelin and the change in PYY. Using pooled data for forward stepwise linear regression, all variables that were significant in bivariate correlations were entered into the prediction model in addition to variables that have previously been reported to be associated with changes in ghrelin, such as body weight, BMI, fat free mass, and energy availability. The strongest predictor of change in ghrelin was change in body weight ($R^2=0.255$, adjusted $R^2=0.216$, $p=0.020$).

![Figure 2](image)

**Figure 2**

Pre-, mid- and post-time points for fasting total ghrelin reveal a significant ($p=0.025$) time by experimental group interaction using an ANOVA with repeated measures. A post hoc analysis using $t$ tests employing the Bonferroni correction factor reveal that the weight loss group has a significant ($p=0.001$) increase in ghrelin concentrations from pre- to post. Values are expressed as mean ± SD. * $p < 0.0167$, Pre- vs. Post.
Table 3. Bivariate correlations for changes in PYY and ghrelin following 3-month diet and exercise program.

<table>
<thead>
<tr>
<th></th>
<th>Δ PYY</th>
<th></th>
<th>Δ ghrelin</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R value</td>
<td>p value</td>
<td>R value</td>
<td>p value</td>
</tr>
<tr>
<td>Changes in body composition</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ body weight (kg)</td>
<td>0.178</td>
<td>0.429</td>
<td>-0.482 *</td>
<td>0.023</td>
</tr>
<tr>
<td>Δ BMI (kg/m²)</td>
<td>0.191</td>
<td>0.395</td>
<td>-0.466 *</td>
<td>0.029</td>
</tr>
<tr>
<td>Δ fat mass (kg)</td>
<td>0.269</td>
<td>0.226</td>
<td>-0.323</td>
<td>0.143</td>
</tr>
<tr>
<td>Δ fat free mass (kg)</td>
<td>-0.128</td>
<td>0.569</td>
<td>-0.430 *</td>
<td>0.046</td>
</tr>
<tr>
<td>Changes in Energy Balance</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ dietary intake (kcal/day)</td>
<td>0.052</td>
<td>0.826</td>
<td>-0.369</td>
<td>0.109</td>
</tr>
<tr>
<td>Δ RMR (kcal/day)</td>
<td>0.185</td>
<td>0.434</td>
<td>-0.201</td>
<td>0.395</td>
</tr>
<tr>
<td>Δ energy availability (kcal/kg•FFM)</td>
<td>-0.118</td>
<td>0.611</td>
<td>-0.474 *</td>
<td>0.030</td>
</tr>
</tbody>
</table>

* Significant correlations (p<0.05); BMI, Body Mass Index; RMR, Resting metabolic rate; FFM, Fat-free Mass.
DISCUSSION

In the current study we hypothesized that non-obese women completing an approximately 3 month diet and exercise training weight loss intervention would experience decreased fasting total PYY and increased fasting total ghrelin concentrations consistent with a plausible compensatory role of these peptides to regulate long term energy balance in response to a reduction in body weight. We implemented a well
controlled and intensive exercise and diet intervention that produced significant changes in key variables associated with aerobic fitness, energy balance, and body composition. In contrast to our hypothesis, while total ghrelin concentrations were elevated with weight loss, total PYY concentrations remained unchanged. The results of this study suggest that over longer time frames, fasting circulating ghrelin is sensitive to changes in body weight and energy availability \textit{per se}, but fasting total PYY appears to not play a role in the modulation of body weight or energy balance over this same duration in non-obese healthy women. Although acute changes in energy availability can change ghrelin and PYY (4, 28), our fasting samples were obtained at least 12-15 hours after the last exercise bout, and so would appear to reflect chronic changes in these peptides. We also hypothesized that exercise in the absence of weight loss would not have a significant effect on fasting total ghrelin or total PYY. Indeed, in our group that experienced significant increases in fitness in the absence of weight loss, we found that exercise itself had no impact on fasting total ghrelin or total PYY concentrations. Although some reports have suggested that ghrelin and PYY may play reciprocal roles in the modulation of energy balance, we could find no evidence of an association between the changes in the peptides during weight loss in our subjects. This is the first study to date to demonstrate these results in a healthy (not obese, not anorexic, or not amenorrheic) population.

As confirmed in numerous previous reports, our long-term intervention demonstrated a clear correlation between weight loss and increases in circulating ghrelin (5, 20, 29, 30). Moreover, the current long-term intervention suggests that changes in ghrelin are related to changes in energy availability. To this end, short-term
manipulations in food intake and exercise expenditure have previously demonstrated a close relationship between energy availability and ghrelin (4, 28). Borer and colleagues (4) manipulated energy intake and exercise energy expenditure during an eleven hour time course in healthy postmenopausal women and demonstrated that ghrelin concentrations respond rapidly to short-term changes in energy availability. Interestingly, Hagobian and colleagues (28) imposed an exercise intervention while manipulating energy intake for four days in men and women and demonstrated that decreases in energy availability causes an increase in acylated ghrelin concentrations in women, but not men. This study indicates that women may be more sensitive to low energy availability and the increases in acylated ghrelin may increase appetite and food intake, in an attempt to maintain bodyweight. The current study adds to the understanding of ghrelin, such that, changes in ghrelin concentrations are also associated with long-term changes in energy availability and changes in body weight, suggesting that ghrelin is clearly sensitive to short- or long-tem changes in energy availability and that ghrelin is involved in short- and long-tem body weight regulation.

Interestingly, Ravussin et al. (26) demonstrated that a negative energy balance over a 100 day period in an absence of any changes in body weight causes an increase in circulating ghrelin concentrations, indicating that the changes in ghrelin can occur in the absence of weight loss and that the alterations in ghrelin concentrations are caused by the changes in energy availability and not a result of a change in body weight. Nutrient sensing mechanisms in the hypothalamus are able to sense the changes in energy balance and modulate energy homeostasis by regulating food intake (31). Through these neuroendocrine mechanisms, small changes in energy availability appear to alter
circulating ghrelin concentrations, attempting to regulate food intake and potentially
energy expenditure. Since ghrelin is involved in both the long-term and short-term
regulations of appetite (32), ghrelin is likely a signal modulated by even small changes in
energy availability involved in the body’s attempt to regulate long-term energy
homeostasis. In support of this idea, we confirmed a significant association between the
change in ghrelin and the change in energy availability in our subjects.

While the current study does not explore the compensatory changes that may
have occurred following the elevated ghrelin concentrations, i.e. elevated food intake or
decreased energy expenditure that would have occurred to compensate for the elevations
in ghrelin following the energy deficit, a well controlled weight loss study with a follow-
up period exploring the consequences of elevated ghrelin (food intake and/or changes in
body weight) should be explored in the future. Interestingly, higher ghrelin in response
to short-term exercise does not seem to increase actual energy intake (33, 34). The latter
finding may suggest that chronic exercise training induced increases in ghrelin may not
lead to compensatory increases in food intake but such studies have not been conducted.

The limitations to this study include that total ghrelin and total PYY were
measured, as opposed to active ghrelin and PYY3-36. In many (11-13, 35, 36) but not all
(37) studies evaluating fasting PYY concentrations, total PYY (PYY1-36 + PYY3-36) is
measured, presumably, because PYY1-36 is more abundant during the fasting state
contributing to 63% of circulating PYY. PYY3-36 is the more potent form, however, both
isoforms exert significant, anorexigenic effects through the binding to NPY/AgRP
neurons in the arcuate nucleus of the hypothalamus to inhibit gastric emptying and
decrease food intake (38-40). Both acylated ghrelin and des-acylated ghrelin circulate in
the blood, while acylated ghrelin is the biologically active form of the hormone (41), however, to date, the majority of weight loss studies have evaluated total ghrelin (acylated ghrelin + des-acylated ghrelin) (5, 6, 8, 20, 29, 30), and the exact endocrine function of des-acylated ghrelin in unknown. A second limitation is that only fasting concentrations of ghrelin and PYY were measured. We have previously reported 24 hour circulating ghrelin concentrations before and after weight loss in non-obese women and found that after a diet and exercise program ghrelin concentrations were elevated after weight-loss when assessed at baseline, lunch, dinner, and during the nocturnal rise (30). Additionally, while fasting ghrelin is correlated with the ghrelin nadir following a meal, fasting PYY concentrations have an even stronger correlation with the PYY post meal peaks (42), indicating that if there is no change in baseline PYY following a weight loss intervention, then the post meal PYY concentrations will likely not be altered compared to pre-intervention. In our laboratory, we have also confirmed strong correlations between fasting PYY and the post prandial peak in PYY in both lunch and dinner, suggesting that fasting PYY relates directly to PYY concentrations after a meal (unpublished data). A third limitation to this study is the small sample size and even though a sample size calculation with 80% power was completed, there is still a 20% chance of a type II error. Additionally, although our intervention was successful in terms of manipulating dietary intake and increasing exercise, an initial analysis based on our original groups produced nonsignificant results for PYY and ghrelin. When subject groups were reorganized according to body weight changes, only ghrelin was shown to increase significantly. Although the latter finding might be explained by varying subject compliance to our diet and exercise prescriptions resulting in cross-over between
groups, our findings should be corroborated by larger studies that are sufficiently
powered to allow for an intent to treat analysis. Finally, another limitation is that there
were initial group differences in baseline body composition. The baseline differences in
the three groups were minimized by analysis of covariance; however, the physiological
impact of differences in baseline body composition may still have impacted the results of
the current study.

In summary, our findings suggest that over longer time frames, circulating ghrelin
is sensitive to changes in body weight and energy availability per se, but fasting total
PYY appears is not involved in the modulation of body weight or energy balance in non-
obese healthy women; therefore circulating PYY concentration are an unlikely candidate
for the regulation of long-term energy balance in healthy subjects.
REFERENCES

7. Leidy HJ, Williams NI. Meal energy content is related to features of meal-related ghrelin profiles across a typical day of eating in non-obese premenopausal women. Hormone and metabolic researchHormon- und StoffwechselforschungHormones et metabolisme. 2006 May;38(5):317.
CHAPTER IV

Scheid JL, De Souza MJ, Hill BR, Leidy HJ, and NI Williams. Decreased luteinizing hormone pulse frequency is associated with elevated twenty-four-hour ghrelin after calorie restriction and exercise training in premenopausal women.

ABSTRACT

Context. Elevated ghrelin has been shown to be associated with reduced luteinizing hormone (LH) pulsatility in men, Rhesus monkeys, and rats. We previously reported that 24 hour ghrelin concentrations are elevated in women following a 3 month exercise and diet program leading to weight loss.

Objective. We investigated whether the elevations in ghrelin following a ~3 month exercise and diet program leading to weight loss are associated with a decrease in LH pulsatility.

Design. A non-exercising control group (Control, n=5) or a diet and exercise group (Energy Deficit, n=16)

Setting. Clinical research center.

Patients. Sedentary women (age, 18-24 years; BMI 18-25 kg/m²)

Intervention. The Control group consumed a controlled diet that matched energy needs, while energy intake in the Energy Deficit group was reduced from baseline energy requirements and supervised exercise training occurred five times per a week.

Main Outcome Measure. The main outcome measures were serum total ghrelin concentrations and LH pulsatility.

Results. Significant decreases in body weight (-3.0± 0.6 kg), body fat (- 2.9±0.4 kg) and 24 hour LH pulse frequency (-0.18±0.08 pulses/hour) and a significant increase in 24
hour mean ghrelin were observed in only the Energy Deficit group. The pre-post change in LH pulse frequency was negatively correlated with the change in mean 24 hour ghrelin (R=-0.485, p=0.030) and the change in peak ghrelin at lunch (R=-0.518, p=0.019).

Conclusions. Elevated total ghrelin concentrations are associated with the suppression of LH pulsatility in premenopausal women and may play a role in the suppression of reproductive function weight loss.

INTRODUCTION

Ghrelin is an orexigenic hormone secreted by distinct endocrine cells of the stomach called X/A-like cells or ghrelin cells (1, 2). Peripherally produced ghrelin is able to cross the blood brain barrier and regulate neuropeptide Y (NPY) and agouti-related protein (AgRP) release from the arcuate nucleus in the hypothalamus (3). Additionally, ghrelin signals are transmitted though the vagus nerve to also stimulate the release of NPY/AgRP (4). Ghrelin activates ghrelin receptors in the arcuate nucleus to upregulate the release of NPY and AgRP, and suppresses the activation of the pro-opiomelanocortin (POMC) and cocaine- and amphetamine regulated transcript (CART), leading to an increase in hunger and food intake (3, 5). Through these central mechanisms, ghrelin has been proposed to play a role in short-term energy homeostasis (5). Interestingly, ghrelin may also impact reproductive function through its central actions on hypothalamic neurons in the arcuate nucleus causing indirect alterations on gonadotropin-releasing hormone (GnRH) neurons (6, 7).

Women with anorexia nervosa and exercising women with functional hypothalamic amenorrhea experience suppressed reproductive function and elevated ghrelin concentrations (8-12). Animal models have demonstrated a direct relationship
between elevated ghrelin and reproductive suppression (13-15). Only one investigation to date has directly explored the relationship between elevated ghrelin and reproductive function in women (16) and failed to demonstrate any effect on luteinizing hormone (LH) pulsatility. However, other investigators (17) have demonstrated that pulsatile LH secretion is suppressed in men following prolonged ghrelin administration, presumably indicating that chronic elevation in ghrelin is necessary to elicit a suppression of LH pulses.

We previously conducted a 3 month randomized controlled trial to examine the impact of caloric restriction combined with exercise on menstrual cyclicity and reproductive function in premenopausal, previously untrained women. We found that fasting and 24 hour total ghrelin concentrations were elevated in the experimental group that lost weight during the intervention when compared to subjects who did not exercise and maintained body weight (18, 19). Although we have documented that our intervention produced menstrual disturbances (20), we have not examined the impact of our intervention on LH pulsatility. To date, there are no studies that have evaluated if the chronic elevation in ghrelin concentrations following diet- and exercise-associated weight loss is accompanied by and/or associated with a decrease in LH pulsatility.

The purpose of this study is to investigate if elevations in 24 hour circulating ghrelin concentrations following a ~3 month exercise and diet program associated with diet- and exercise-induced weight loss are associated with a decrease in LH pulsatility in premenopausal women. We hypothesized that 1) the women who experience an energy deficiency will demonstrate a significant decrease in LH pulsatility compared to the women in the Control group who will demonstrate no significant changes in LH
pulsatility and 2) there will be a significant negative correlation between changes in ghrelin concentration and changes in LH pulse frequency.

SUBJECTS AND METHODS

Screening

This study was part of a larger, prospective study designed to assess changes in endocrine and reproductive function in response to a controlled feeding and exercise intervention. The intervention was implemented in sedentary women to emulate exercise and restrictive eating patterns in which many young women engage. Subjects included non-smoking, healthy, non-exercising (< 1 hour/week purposeful exercise) women ages 18-30, 15-30% body fat and BMI between 18-25 kg/m^2. Exclusion criteria included any evidence of disordered eating or history of an eating disorder, loss/gain of a significant amount of weight (±2.3kg) in the past year, or use of hormonal contraceptives or medication that may alter metabolic hormones. Each subject signed an informed consent approved by the Biomedical Institutional Review Board of The Pennsylvania State University.

Subjects provided information regarding demographics, medical history, menstrual history, and physical activity along with eating attitudes questionnaires. A fasting blood sample was obtained for analysis of a complete blood count, basic chemistry panel, and to rule out abnormal pituitary function or metabolic diseases. Psychological stability and the absence of eating disorders or risk of developing an eating disorder were established in an interview under the supervision of a clinical psychologist. Subjects met with a general clinical research center registered dietician to ensure absence of aberrant dietary habits and suitability for a controlled feeding study. Documentation
of 2-3 ovulatory menstrual cycles prior to the study was performed with measurements of mid-luteal phase serum progesterone and the mid-cycle urinary LH surge (First Response, Tambrands, Inc.).

**Subject groupings**

Following a screening and a baseline monitoring period of 4 weeks, subjects completed a 3-month diet and exercise intervention. Subjects included in this sub-study were the subjects who 1) completed the 24 hour blood sampling both pre- and post-intervention, and 2) had been randomly assigned to either a non-exercising Control group (n=5), who consumed enough calories to maintain weight, or to the Energy Deficit group (n=16), who were prescribed and provided reductions in food calories and exercised to achieve a negative energy balance ranging from -30% to -60% compared with baseline energy needs. One subject in the non-exercising Control group was not included because they lost over 5kg and were not considered compliant to the protocol. One subject in the Energy Deficit group was not included because of documentation of a luteal phase defect during their control menstrual cycle.

**Dietary intake during the intervention**

Dietary intake was controlled throughout the intervention (18). At baseline, each subject’s daily energy requirement to maintain a stable body weight was determined through estimates of energy expenditure followed by a week-long “calibration” period allowing for adjustments in the dietary prescription if body weight fluctuated. To estimate total energy expenditure, resting metabolic rate was measured using indirect calorimetry, and this value was added to the total calories expended during a 24-hour
period as assessed by a triaxial accelerometer (RT3 accelerometer; Stayhealthy, Monrovia, CA) (18, 19).

After the baseline period, the Control group continued to consume the weight maintenance calorie level estimated from the 7-day calibration period. The Energy Deficit group was provided fewer calories (mean ± standard error -25 ± 8%) than those required to maintain initial body weight, and began supervised exercise training. The diet was comprised of 55% carbohydrate, 30% fat, and 15% protein (18, 19).

**Exercise training during the intervention**

Exercise was supervised throughout the entire study (18, 19). While the Control group did not perform any exercise, the Energy Deficit group performed aerobic exercise five times per week at 70%-80% of maximum heart rate as determined from tests of maximal aerobic capacity. The total amount of calories expended during each exercise session was measured using the OwnCal feature on the Polar S610 heart rate monitor (Polar Electro Oy, Kempele, Finland).

*Fitness and Body Composition*

Body composition was determined using hydrostatic weighing, and maximal aerobic capacity was determined using indirect calorimetry during the baseline and post-intervention time-points according to previously published methods (18, 19).

**Twenty-four-hour Repeated Blood Sampling**

Subjects reported to the General Clinical Research Center at 0730 h subsequent to an overnight fast and abstaining from exercise for 24 hours. An intravenous catheter was inserted into a forearm vein. Blood samples were obtained every 10 minutes for 24 hours.
both before and after the 3 month weight loss intervention. Subjects remained in a supine position with their upper body and head slightly elevated. All postural changes were recorded. Meals were provided at 0900 h (breakfast), 1200 h (lunch), 1800 h (dinner), and 2100 h (snack), and subjects consumed these meals within 30 minutes. Total calories over the day represented 85% of each subject’s weight maintenance intake to account for negligible physical activity during the procedure. Each subject consumed a 500-calorie dinner, and the rest of the daily calories were distributed over the remaining meals (43% at breakfast, 49% at lunch, and 4% for the snack). The macronutrient composition of the food over the entire day averaged 55% carbohydrate, 30% fat, and 15% protein and was not significantly different among the three meals (19).

**Total Ghrelin**

Total ghrelin was measured in duplicate in serum samples from the 24-hour repeated blood sampling procedure hourly from 0800 to 1000 h, every 20 minutes from 1000 to 2000 h and hourly from 2000 to 0800 h using the Linco Research radioimmunoassay kit (St. Charles, MO). Assay sensitivity was 100 pg/mL. The intra-assay and inter-assay coefficients of variation for the high control were 2.7% and 16.7%, respectively; the intra-assay and inter-assay coefficients of variation for the low control were 1.2% and 14.7%, respectively. All samples from a given subject were included in the same assay.

**Luteinizing Hormone**

Luteinizing hormone was measured in serum samples from the 24-hour repeated blood sampling procedure every 10 minutes from 0800 to 0800 h using the Siemens
Immulite kit (Deerfield, IL). Assay sensitivity is 0.1 mIU/mL. The intra-assay and inter-assay coefficients of variation are 5.7% and 12.3%.

Data Analysis

Luteinizing Hormone Pulse Analysis. The time series of the 24 hour LH concentrations was analyzed for pulse frequency, peak amplitude, peak height, and 24 hour mean LH using the pulse detection algorithm Cluster (CLUSTER 8) (21). A 2 x 1 pulse configuration was used with a $t$ statistic value of 2.0 for both upstroke and downstroke. Missing data was linearly interpolated between the two adjacent values. The LH variables of interest included: mean LH, LH pulse frequency, maximal peak amplitude, mean interval between LH peaks, and LH area under the curve. LH area under the curve was calculated using the computer program Cluster (21) and was defined as the product of the mean peak amplitude and the time of the interval.

Ghrelin Analysis. Twenty-four-hour mean ghrelin was represented as the average of all ghrelin concentrations (pg/ml) observed in the 24-hour analysis. Total ghrelin area under the curve was calculated using the trapezoidal rule. Meal peaks were defined as the highest ghrelin concentration (pg/ml) that occurred prior to the meal administration. Meal response averages were the mean of ghrelin concentrations (pg/ml) from 2 hours prior through 2 hours after the meal.

Statistical Analysis. Data screening prior to analysis involved outlier detection and tests of normality. A paired t-test was used to determine whether significant within group changes occurred in LH pulse frequency and ghrelin from pre- to post-intervention. Wilcoxon signed Rank Tests were used when the data were not normally distributed. For comparisons of the changes between groups, independent t-tests on change scores for LH
pulse parameters and ghrelin parameters were employed. Mann-Whitney U Tests were used when the data were not normally distributed. Pearson correlation coefficient analyses were performed to examine relationships between LH and ghrelin when both groups were combined. Stepwise linear regression was used to determine if the change in ghrelin was an independent predictor of change in LH pulse frequency. To explore the predictors of LH pulse frequency, change in body weight, change in mean 24 hour ghrelin, change in lunch peak, and change in body fat were entered in to the stepwise linear regression model. All analyses were performed using SPSS software (version 16.0; SPSS, Inc., Chicago, IL). All data were reported as mean ± sem.

Sample size was based previously published data (22) demonstrating a significant correlation of serum ghrelin and serum LH in male rats. Although a population correlation of 0.724 was reported in Abou et al. (22), we chose a conservative value of 0.6. Thus, using a bivariate correlation, using two tails, a population correlation of 0.6, an alpha error probability of 0.05, and a power of 0.80, a total sample size of 19 was needed.

RESULTS

Baseline descriptive data for subjects in Control and Energy Deficit groups are shown in Table 1. The Energy Deficit group had a higher initial height, body weight, and fat mass, but were similar with respect to age, BMI, fat-free mass, and initial fitness compared to the Control group.

Changes in body composition and fitness are shown in Table 1. As expected, the Energy Deficit group had a significant decrease in body weight, BMI, body fat percentage and fat mass, while the Control group demonstrated no changes in body
weight, BMI, body fat percentage or fat mass. Additionally, fitness levels increased (p=0.001) in the Energy Deficit group. A one-way ANOVA demonstrated that the only change in body composition that was significantly different between the groups was fat mass which was greater in the Energy Deficit group compared to the Control group (p=0.038). Body weight changes ranged from +1.1 kg to -7.55 kg in the Energy Deficit group, +0.5 kg to -1.25 kg in the Control group.

Prescribed calorie intake and actual calorie intake varied less than 35 calories per week during the intervention, and no differences in this parameter existed among groups. Final macronutrient intake was in accordance with that prescribed i.e., of 55% carbohydrates, 30% fat, and 15% protein with no differences between groups. With respect to compliance to the exercise training protocol, 15 of the 16 exercising women (94%) consistently reached their prescribed exercise calorie level per week and target exercise intensity level (70–80% of maximal heart rate).
Table 1. Subject characteristics during the pre- and post-intervention in the Control and the Energy Deficit groups.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 5)</th>
<th>Energy Deficit (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>21.2±0.7</td>
<td>N/A</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>159.1±1.1</td>
<td>N/A</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>52.1±0.9</td>
<td>50.9±1.1</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>20.6±0.5</td>
<td>20.1±0.7</td>
</tr>
<tr>
<td>Fat mass (%)</td>
<td>24.6±2.2</td>
<td>23.2±1.8</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>12.8 ±1.1</td>
<td>11.8±0.9</td>
</tr>
<tr>
<td>Fat-free mass (kg)</td>
<td>39.3±1.4</td>
<td>39.0±3.0</td>
</tr>
<tr>
<td>VO₂ max (ml·kg·min⁻¹)</td>
<td>36.5±3.0</td>
<td>38.0±1.3</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM.

Independent T-tests were used to compare change in body composition and fitness.

Paired t-tests were used to compare body composition and fitness pre- vs. post-study.

*Paired t-test, Pre- vs. Post-study, p < 0.05, †Independent t-test, Control vs. Energy Deficit, p<0.05

Ghrelin concentrations both pre- and post-intervention are presented in Table 2.
Table 2. Comparison of pre- and post-intervention ghrelin concentrations, and meal related ghrelin concentrations in the Control and the Energy Deficit group.

<table>
<thead>
<tr>
<th>Ghrelin Concentrations</th>
<th>Control (n = 5)</th>
<th>Energy Deficit (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>Mean 24 hr (pg/ml)</td>
<td>1,616±122</td>
<td>1,634±178</td>
</tr>
<tr>
<td>Area under the curve</td>
<td>37,663 ±2,885</td>
<td>37,953±3,752</td>
</tr>
<tr>
<td>Meal peaks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breakfast peak (pg/ml)</td>
<td>1,780±175</td>
<td>1,908±303</td>
</tr>
<tr>
<td>Lunch peak (pg/ml)</td>
<td>1,764±170</td>
<td>1,774±238</td>
</tr>
<tr>
<td>Dinner peak (pg/ml)</td>
<td>1,929±153</td>
<td>1,953±250</td>
</tr>
<tr>
<td>Meal response averages *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breakfast response average (pg/ml)</td>
<td>1,559±182</td>
<td>1,514±238</td>
</tr>
<tr>
<td>Lunch response average (pg/ml)</td>
<td>1,511±173</td>
<td>1,514±199</td>
</tr>
<tr>
<td>Dinner response average (pg/ml)</td>
<td>1,655±103</td>
<td>1,709±198</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM.

Independent T-tests were used to compare change in Lunch peak and Breakfast response average.

Mann-Whitney U Tests were used to compare the change in Mean 24 hr, Area under the curve, Breakfast peak, Dinner peak, Lunch response average, and Dinner response average. Paired t-tests were used to compare all ghrelin concentrations pre- vs. post-study.† Paired t-test, Pre- vs. Post-study, p < 0.05

* Meal response averages were the mean of ghrelin concentrations (pg/ml) from 2 hours prior through 2 hours after the meal.
Examples of individual 24 hour ghrelin profiles are presented in Figure 1. Mean 24 ghrelin concentrations, 24 hour ghrelin area under the curve, breakfast peak, lunch peak, dinner peak, breakfast response average, lunch response average, and dinner response average were all similar (p>0.05) between the groups before the intervention. Paired t-test demonstrated that the Energy Deficit group had a significant increase in mean 24 ghrelin concentrations (p=0.002), 24 hour ghrelin area under the curve (p=0.005), lunch peak (p<0.001), dinner peak (p=0.001), breakfast response average (p=0.030), lunch response average (p=0.004), and dinner response average (p=0.004), while the Control group did not demonstrate any changes in ghrelin characteristics.
**Figure 1.** Examples of individual LH 24 hour profiles during the pre- and post-intervention. Panel A) Example subject #1 was in the Control group, lost 0.1 kg of body weight, increased LH pulse frequency by 0.02 pulses per hour, and increased mean 24 hour ghrelin by 116 pg/ml. Panel B) Example subject #2 was in the Energy Deficit group, lost 3.3 kg of body weight, decreased LH pulse frequency by 0.75 pulses per hour, and increased mean 24 hour ghrelin by 239 pg/ml. Panel C) Example subject #3 was in the Energy Deficit group, lost 6.3 kg of body weight, decreased LH pulse frequency by 0.89 pulses per hour, and increased mean 24 hour ghrelin by 1072 pg/ml. LH=Luteinizing hormone.
LH characteristics for both pre- and post-intervention 24 hour sampling periods are presented in **Table 3**. Examples of individual 24 hour LH profiles are presented in **Figure 1**. Mean LH, LH pulse frequency, maximal peak amplitude, mean interval between LH peaks, and LH area under the curve were all similar (p>0.05) between the groups before the intervention. Paired t-tests demonstrated that LH pulse frequency decreased (p=0.047) and LH area under the curve increased (p=0.021) in the Energy Deficit group, while no significant change was seen in any LH parameter from pre- to post-intervention in the Control group.
Table 3. Luteinizing Hormone Characteristics during the pre- and post-intervention in the Control and the Energy Deficit group.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Energy Deficit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 5)</td>
<td>(n = 16)</td>
</tr>
<tr>
<td>Mean LH (mIU/mL)</td>
<td>5.2±0.7</td>
<td>5.1±0.4</td>
</tr>
<tr>
<td></td>
<td>5.7±1.0</td>
<td>4.7±0.6</td>
</tr>
<tr>
<td></td>
<td>0.5±0.5</td>
<td>-0.5±0.5</td>
</tr>
<tr>
<td>LH Pulse Frequency</td>
<td>0.76±0.11</td>
<td>0.81±0.06</td>
</tr>
<tr>
<td>(pulses/hour)</td>
<td>0.75±0.11</td>
<td>0.64±0.08*</td>
</tr>
<tr>
<td></td>
<td>0.00±0.10</td>
<td>-0.18±0.08*</td>
</tr>
<tr>
<td>Maximal Peak Amplitude</td>
<td>10.3±0.7</td>
<td>9.6±0.6</td>
</tr>
<tr>
<td>(mIU/mL)</td>
<td>10.4±0.9</td>
<td>10.4±1.0</td>
</tr>
<tr>
<td></td>
<td>0.1±0.2</td>
<td>0.9±0.7</td>
</tr>
<tr>
<td>Mean Interval</td>
<td>85.7±12.3</td>
<td>86.0±13.1</td>
</tr>
<tr>
<td>between LH Peaks</td>
<td>86.3±10.7</td>
<td>108.7±21.5</td>
</tr>
<tr>
<td>(mins)</td>
<td>0.6±13.8</td>
<td>23.0±30.3</td>
</tr>
<tr>
<td>LH area under the</td>
<td>127.7±29.0</td>
<td>143.9±28.2</td>
</tr>
<tr>
<td>curve</td>
<td>143.9±28.2</td>
<td>16.2±29.2</td>
</tr>
<tr>
<td></td>
<td>109.9±14.1</td>
<td>177.5±28.2*</td>
</tr>
<tr>
<td>Cycle Day of Testing</td>
<td>4.6±1.3</td>
<td>5.3±0.4</td>
</tr>
<tr>
<td>(day)</td>
<td>6.0±1.3</td>
<td>8.7±2.3</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM. LH=Luteinizing hormone
Independent T-tests were used to compare change in Mean LH, LH Pulse Frequency, Maximal Peak Amplitude, LH area under the curve, and Cycle Day of Testing. Mann-Whitney U Tests were used to compare the change in Mean Interval between LH Peaks. Paired t-tests were used to compare Mean LH, LH Pulse Frequency, Maximal Peak Amplitude, and LH area under the curve pre- vs. post-study. Wilcoxon signed Rank Tests were used to compare Mean Interval between LH Peaks pre- vs. post-study.*Paired t-test ,Pre- vs. Post-study, p < 0.05

Pearson correlations between change in LH pulse frequency, and change in ghrelin concentrations and changes in body composition in all subjects are presented in Table 4. There were negative correlations between the change in LH pulse frequency
and the change in mean 24 hour ghrelin (Figure 2), 24 hour ghrelin area under the curve, ghrelin lunch peak (Figure 2), ghrelin dinner peak, ghrelin breakfast response average, ghrelin lunch response average, and ghrelin dinner response average. There were no correlations between change in LH pulse frequency and change in BMI, fat mass or fat-free mass. However, a positive correlation between changes in LH pulse frequency and changes in body weight was also demonstrated.

Table 4. Bivariate correlations of change in LH pulse frequency (n=21).

<table>
<thead>
<tr>
<th>Change in LH Pulse Frequency</th>
<th>R value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Changes in ghrelin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change in mean 24 hr ghrelin (pg/ml)</td>
<td>-0.485</td>
<td>0.030*</td>
</tr>
<tr>
<td>Change in ghrelin area under the curve</td>
<td>-0.470</td>
<td>0.049*</td>
</tr>
<tr>
<td>Change in ghrelin breakfast peak (pg/ml)</td>
<td>0.034</td>
<td>0.888</td>
</tr>
<tr>
<td>Change in ghrelin lunch peak (pg/ml)</td>
<td>-0.518</td>
<td>0.019*</td>
</tr>
<tr>
<td>Change in ghrelin dinner peak (pg/ml)</td>
<td>-0.484</td>
<td>0.031*</td>
</tr>
<tr>
<td><strong>Changes in body composition</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change in body weight (kg)</td>
<td>0.435</td>
<td>0.049*</td>
</tr>
<tr>
<td>Change in BMI (kg/m²)</td>
<td>0.426</td>
<td>0.054</td>
</tr>
<tr>
<td>Change in fat mass (kg)</td>
<td>0.323</td>
<td>0.154</td>
</tr>
<tr>
<td>Change in fat free mass (kg)</td>
<td>0.282</td>
<td>0.229</td>
</tr>
</tbody>
</table>

*Significant correlations (p<0.05)
Figure 2. Relationship between the change in LH pulsatility and the change in ghrelin lunch peak, and change in LH pulsatility and change in mean 24 hour ghrelin, from pre- to post intervention (n=21).
To explore the strongest predictors of LH pulse frequency using stepwise linear regression, all variables that were significant in bivariate correlations were entered into the prediction model in addition to variables that have previously been reported to be associated with changes in LH pulse frequency, such as body weight, BMI, fat free mass, mean ghrelin concentration and ghrelin meal peaks. The strongest predictor of change in LH pulse frequency was change in ghrelin lunch peak ($R^2=0.268$, adjusted $R^2=0.228$, $p=0.019$).

**DISCUSSION**

In the current study we implemented a well-controlled, intensive exercise and diet intervention in 21 premenopausal, sedentary women and demonstrated that 1) the women in the Energy Deficit group experienced a decrease in LH pulsatility, 2) the women in the Energy Deficit group experienced a increase in mean 24 hour total ghrelin concentrations and several meal-related ghrelin parameters, 3) increases in circulating ghrelin concentrations occurred as a result of a negative energy balance and subsequent weight loss, 4) changes in ghrelin were associated with decreases in LH pulsatility, and 5) change in ghrelin lunch peak was the strongest independent predictor of LH pulse frequency (demonstrated using regression). We speculate that changes in ghrelin may be associated with changes in LH pulse frequency independent of changes in body weight. Perhaps this is beneficial in that ghrelin can change more rapidly than body weight and perhaps is therefore a more sensitive signal of changes in energy balance than are changes in body weight. Additionally, total body weight changes during an intervention employing both caloric restriction and exercise to not always agree with calculations of energy deficits because training may stimulate increases in muscle mass, increases in
plasma volume (23), and increases in body water stored with increased glycogen storage (24).

Short term manipulations of energy intake and exercise expenditure, also known as energy availability, have consistently demonstrated that changes energy availability are related to changes LH pulse frequency (25, 26). In men, forty-eight hours of fasting also resulted in a decrease in LH pulse frequency (27). Similarly, in women, two days of fasting resulted in a decrease in LH pulse frequency (28). Our current study demonstrates, prospectively, in a long-term study, controlling both diet and exercise, an association between an energy deficit and a decrease in LH pulse frequency.

In the current study, we also demonstrated that 24 ghrelin concentrations were elevated following an intensive exercise and diet intervention. However, we have previously demonstrated that fasting ghrelin (18) and 24 hour ghrelin concentrations (19) were elevated as a result of caloric restriction combined with exercise. To this end, numerous previous reports have demonstrated a clear association between weight loss and increases in circulating ghrelin (18, 19, 29, 30). Ghrelin is likely a signal modulated by an energy deficit in the body’s attempt to regulate long-term energy homeostasis.

This is the first study to demonstrate a relationship between elevated ghrelin and decreases in LH pulse frequency in premenopausal women following diet- and exercise-associated weight loss. Elevated ghrelin concentrations have been associated with decreases in LH in two pharmacological studies conducted in men (17). Kluge and colleagues (17) demonstrated a decrease in LH pulse frequency and amplitude following four ghrelin injections (50µg) over a 12 hour period. Similarly, Lanfranco and colleagues (31) demonstrated that an 8-hour administration of acylated ghrelin decreased LH
pulsatility. Both of these studies indicate that elevated levels of circulating ghrelin concentrations can inhibit reproductive function by decreasing LH pulse frequency.

In women, Messini and colleagues (16) examined the effect of a single ghrelin injection in normally menstruating women and failed to demonstrate any effect of the acute ghrelin injection on LH indicating that either; 1) one injection of ghrelin was not enough to elicit a suppression of LH pulses, or that 2) elevated ghrelin concentrations do not affect LH pulses in women. The current study is the first to support the concept that elevated ghrelin concentrations may contribute to the suppression of LH pulses in women that are observed in association with a diet and exercise intervention leading to weight loss. However, although changes in ghrelin were an independent predictor of changes in LH, the correlation was rather modest. This is not unexpected, since numerous other metabolic hormones have been shown to act as metabolic signals that can impact GnRH neurons (32, 33).

The underlying mechanism causing elevated ghrelin to down regulate LH secretion from the anterior pituitary is unknown. Ghrelin is likely able to impact LH pulse frequency by altering concentrations of NPY/AgRP and POMC/CART in the arcuate nucleus, either by crossing the blood brain barrier or by signals transmitted by the vagus nerve to affect appetite and, indirectly by impacting the hypothalamic-pituitary-ovarian axis (4, 6). Studies in animals suggest that elevated ghrelin causes alterations in GnRH secretion from the GnRH neurons (14). Lebrethon and colleagues (34) administered ghrelin to male rats and demonstrated a decrease in the GnRH interpulse interval, while Furuta and colleagues (14) have suggested that since ghrelin injected in ovariectomized rats decreases only LH pulse frequency and not LH pulse amplitude,
ghrelin presumably affects LH secretion at the level of the GnRH pulse generator and not the anterior pituitary. The ghrelin receptor, i.e., the growth hormone secretagogue receptor, has been identified in the hypothalamic neurons including the arcuate nucleus and paraventricular nucleus (35). Elevated ghrelin concentrations can cause alterations of neuropeptides including NPY, POMC, and kisspeptin (7, 36). Alterations in these neuropeptides, NPY, POMC, and KiSS-1, then down regulate GnRH secretion leading to a downstream decrease in pituitary release of LH (7, 36-38).

It is important to note that many metabolic signals have been shown to directly, or indirectly affect LH pulsatility (32, 33, 39, 40). For example, there is also evidence to suggest that leptin is involved in regulating the hypothalamic-pituitary-ovarian axis in humans (32, 41, 42). Welt et al. (32) administered human recombinant leptin to eight women with FHA and demonstrated an increase in LH pulsatility; however, leptin 24 hr concentrations were not measured in the current study.

A major limitation to this study is that a cause and effect relationship between ghrelin and the decline in LH pulse frequency cannot be established. However our data add support to the concept that elevated ghrelin concentrations may cause suppression of LH pulses in exercising women who lose weight. A second limitation may be the sample size. However, the small sample size reflects the difficulties in conducting a study with a high degree of subject burden including 24 hour blood draws and a controlled three month intervention that involved both a supervised exercise program and a controlled feeding dietary intervention.

In summary, our findings suggest that the changes in ghrelin associated with an energy deficiency are also associated with the change in LH pulse frequency in
premenopausal women. Understanding the role of ghrelin in regulating the hypothalamic-pituitary-ovarian axis will help to elucidate the mechanism of exercise–associated menstrual disturbances. If ghrelin is directly participating in the down regulation of reproductive function in women with exercise-associated menstrual disturbances, future studies will need to investigate if improving energy status in women with amenorrhea causes a decrease in ghrelin and if this decrease initiates the reversal of amenorrhea.
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Scheid JL, Birch LL, Williams NI, and MJ De Souza. Dietary cognitive restraint is related to peptide YY concentrations in young college-aged women.

ABSTRACT

Acylated Ghrelin and Peptide YY (PYY3-36) are involved in appetite-regulation and energy homeostasis, leading to the control of body weight. Dietary restraint is an eating behavior phenotype manifest as a conscious cognitive control of food intake in order to achieve or sustain a desired body weight. To date, no studies have been conducted that examine postprandial feeding signals such as PYY3-36 and acylated ghrelin in young women with high dietary restraint compared to women with normal dietary restraint. Understanding the patterns of release of acylated ghrelin and PYY3-36 following a meal in young women with high cognitive restraint may help to explain the link between eating behavioral phenotypes and the underlying biological mechanisms related to body weight regulation. The purpose of the current study was to determine if college-aged women (18 to 25 years) with different eating behavioral phenotypes, i.e., high vs normal dietary restraint, differ with respect to circulating concentrations of gastrointestinal hormones during and following a test meal. We hypothesized that women with high dietary cognitive restraint [High CR (score ≥ 13, n=13)] would have elevated active ghrelin and PYY3-36 concentrations and lower glucose and insulin concentrations after a test meal compared to women with normal dietary cognitive restraint [Normal CR (score < 13 (n=30)]. Gastrointestinal hormones were assessed before (-15 and 0 min) and after (10, 15, 20, 30, 60, 90, 120 and 180 min) the consumption of a mixed composition meal (5.0 kcal per kg/body weight). In contrast to our hypothesis, mean PYY3-36 concentrations
(p=0.042), peak PYY<sub>3-36</sub> concentrations (p=0.047) and PYY<sub>3-36</sub> area under the curve (p=0.035) were lower in the High CR group compared to the Normal CR group after controlling for body mass index. No group differences were observed with respect to acylated ghrelin, glucose, or insulin before or after the meal. In conclusion, PYY<sub>3-36</sub> concentrations were suppressed in the women with High CR compared to the women with Normal CR. We speculate that elevated dietary restraint in college-aged women may be a compensatory behavioral mechanism to avoid weight gain that might result from suppressed PYY<sub>3-36</sub> circulating concentrations after a meal.

**INTRODUCTION**

Associations between eating behavior phenotypes and gastrointestinal hormones have only recently been explored in normal weight populations [1, 2]. A common eating behavior phenotype frequently exhibited in young college-aged women is dietary restraint [3]. Dietary cognitive restraint, a concept originally proposed by Herman and Mack [4], refers to a conscious control of food intake in order to achieve or sustain a desired body weight [5, 6]. The Three Factor Eating Questionnaire (TFEQ) measures dietary restraint in addition to two other dimensions of human eating behavior i.e., disinhibition, and hunger [6]. Disinhibition reflects a loss of control of inhibited food intake, while hunger indicates feelings of hunger and related behavioral consequences [6].

Ghrelin and peptide YY (PYY) are both peptides secreted from the gastrointestinal track that are involved in appetite-regulation and the physiological regulation of body weight [7-9]. Both ghrelin and PYY circulate in the blood, cross the blood brain barrier and bind to receptors in the hypothalamus [8, 12, 13]. Acylated ghrelin (the active form of ghrelin) stimulates the release of neuropeptide Y (NPY) and agouti-related protein
(AgRP), from hypothalamic neurons, which stimulate hunger and food intake [10].
PYY₃₋₃₆ (the active form of PYY) activates the Y2 receptors to inhibit the release of NPY and AgRP, while PYY₃₋₃₆ also stimulates the release of pro-opiomelanocortin (POMC) and cocaine- and amphetamine regulated transcript (CART), to decrease hunger and food intake [11, 12]. Additionally, signals are transmitted though the vagus nerve to stimulate the release of NPY/AgRP and impact hunger and food intake [13]. Understanding the patterns of release of acylated ghrelin and PYY₃₋₃₆ following a meal in young women with high cognitive restraint may help to explain the link between eating behavioral phenotypes and the underlying biological mechanisms related to body weight regulation. Further investigation of the role of eating attitudes and behaviors is necessary in this behavioral-metabolic paradigm in order to determine if gastrointestinal hormones are key regulatory factors in long-term over- or under- consumption of food intake.

The relationships between eating behavior phenotypes and gastrointestinal hormone concentrations are not clear. To date, a limited number of studies have been conducted that examine postprandial feeding signals such as PYY₃₋₃₆ and acylated ghrelin in young women with different eating behavior phenotypes [2, 14]. In non-obese men and women, Schur et al. [1] demonstrated a significant positive correlation between dietary restraint and fasting total ghrelin concentrations, indicating that high dietary restraint is associated with elevated ghrelin concentrations. However, to date, postprandial ghrelin response patterns have not been evaluated in women with elevated dietary cognitive restraint. Elevated fasting concentrations of circulating ghrelin and PYY have been previously observed in women with menstrual cycle disturbances [15-19] and an elevated dietary restraint has also been demonstrated to be associated with
menstrual cycle disturbances [3, 20]. In line with these reports, we speculate that elevated ghrelin and PYY may be related to an energy deficiency caused by high dietary restraint in young women.

Gastrointestinal peptides are altered following food intake and thus postprandial measurements are important when investigating meal related peptides. Ghrelin concentrations decrease following food intake, and this decrease in ghrelin is thought to trigger the termination of a meal [21], suggesting that circulating concentrations of ghrelin 10 – 20 min following food intake are important to evaluate when attempting to understand meal termination. However, PYY concentrations increase for several hours after meal termination [21, 22], and we speculate that PYY concentrations released 2 – 3 hours following a meal may be involved in the eating behavior of the next meal or snack. Individual differences in meal frequency could have substantial effects on energy intake [23]. Therefore, measuring PYY concentrations for 2 – 3 hours after a meal may have important physiological relevance with respect to understanding the relationship between PYY and eating behaviors.

Both circulating ghrelin and PYY are altered in obese populations and, to this end, relationships between these gut hormones and BMI have previously been demonstrated [7, 22]. Additionally, eating behaviors, such as elevated dietary restraint and disinhibition have been previously demonstrated to be associated with obesity [6]. These previous findings must be taken into consideration in the current study and any group differences in BMI will need to be addressed, so that relationships between gastrointestinal peptides and eating behaviors can be investigated.
The purpose of the current study was to determine if college-aged women with high versus normal dietary restraint display different feeding signal responses during and for several hours after a test meal. We hypothesized that women with high dietary restraint, as measured by the TFEQ, would have elevated active ghrelin and PYY3-36 concentrations and lower glucose and insulin concentrations after a test meal when compared to women with normal dietary restraint. Additionally, we hypothesized that women with high dietary restraint would have elevated hunger, a lower desire to eat, and lower fullness measured by visual analog scales (VAS) after a test meal when compared with women with normal dietary cognitive restraint.

METHODS

Experimental Design

We studied 43 college-aged women. The Three-Factor Eating Questionnaire [6] was administrated to characterize women by dietary restraint into two distinct eating behavior phenotype groups: (1) women with high dietary cognitive restraint (High CR), defined as a dietary cognitive restraint score ≥ 13 (n=13), and (2) women with normal dietary cognitive restraint (Normal CR), defined as a dietary restraint score < 13 (n=30). A dietary restraint score ≥ 13 indicates the 75th percentile in the current population of young college-aged women. A dietary restraint score ≥ 13 also represents the 75th percentile in other populations of young college-aged women [24] and postmenopausal women [25, 26]. The gastrointestinal peptides acylated ghrelin and PYY3-36, and insulin, glucose, hunger, fullness and desire to eat were the dependent variables of primary interest in this study and were assessed before and several times up to 180 min after the
consumption of a mixed composition meal (carbohydrate 64%, fat 22%, protein 14%) at a dose of 5.0 kcal per kg/body weight.

**Recruitment and Medical Screening**

Young women were recruited from the general population of college-aged women (18-25 yrs) at Penn State University and the surrounding community. Screening procedures included a physical exam, a psychological interview, and questionnaires regarding self-reported medical health history, eating behaviors, and past physical activity. Inclusion criteria were: 1) female, 2) aged 18-25 years, 3) habitual breakfast eater, 4) non-smoker, 5) not using hormonal contraceptives for at least 6 months prior to study, 6) weight stable (+/- 2 kg) for the last six months, 6) not pregnant or lactating, 7) no current clinical diagnosis of an eating or psychiatric disorder, 8) no food sensitivities or allergies to the test meal, and 9) screening CBC: hemoglobin >11.5 mg/dl, hematocrit >35%. Exclusion criteria were: 1) known or suspected metabolic or endocrine disease, 2) use of anti-depressants or other psychiatric medications, 3) use of medications that alter absorption or food intake, 4) medical conditions associated with malabsorption .e.g. Celiac disease or Crohn’s disease. The study was approved by the Penn State University Biomedical Institutional Review Board and all volunteers signed an approved Informed Consent document.

**Dietary Cognitive Restraint**

Dietary restraint score was assessed as one of three subscales of the TFEQ. The TFEQ is a 51-item questionnaire that measures three dimensions of human eating behavior: (1) dietary cognitive restraint, (2) disinhibition, and (3) hunger [6]. The dietary restraint scores include 21 items, and scores range from 0 to 21, and higher scores reflect
higher levels of conscious control of food intake. Disinhibition scores range from 0 to 16 and higher scores reflect a loss of control of food intake. Hunger scores range from 0 to 14 and higher scores indicate greater hunger. Additionally, we measured rigid and flexible control of eating behavior, two subscales of the TFEQ [27]. Rigid control scores indicate an ‘all or nothing’ approach to eating and dieting, while flexible control indicates a more moderate approach to eating and dieting [27, 28]. These subscales of the TFEQ provide more information regarding specific dietary restraint patterns. Rigid control is associated with high disinhibition and more frequent binge eating, while high flexible control is associated with low disinhibition and less binge eating in obese and non-obese women [27, 29, 30].

The Dutch Eating Behavior Questionnaire (DEBQ) also measures dietary restraint and disinhibition and consists of three factors: (1) dietary restraint, or cognitive control of eating, (2) emotional disinhibition, or loss of control over eating due to emotions and (3) external disinhibition, or loss of cognitive control of eating due to the presence of food [31]. The second factor (emotional disinhibition) may be divided into two additional factors: (a) loss of cognitive control of eating due to specific emotions (i.e. if an individual is upset due to something bad that just happened), or (b) loss of cognitive control of eating due to diffused emotions, (i.e. eating due to boredom in general). The combination of factors (2) and (3), emotional disinhibition and external disinhibition, define the entire construct of dietary disinhibition. The DEBQ was used to corroborate the TFEQ measure of restraint and also further describe the eating behaviors in the women in the High CR group compared to the Normal CR group.
Body Dissatisfaction and Drive for Thinness

Body dissatisfaction and drive for thinness were assessed as part of the Eating Disorder Inventory-2 (EDI-2). The EDI-2 is a 91-item self-report measure of multidimensional symptom clusters commonly associated with eating disorders [32]. The EDI-2 is comprised of 3 subscales assessing attitudes and behaviors concerning eating, weight, and shape (drive for thinness, bulimia, body dissatisfaction), 5 subscales assessing organizing constructs or psychological traits clinically relevant to eating disorders (ineffectiveness, perfectionism, interpersonal distrust, interoceptive awareness, maturity fears), and 3 provisional subscales (asceticism, impulse regulation, social insecurity). Body dissatisfaction and drive for thinness were used to describe the eating attitudes in the High CR group compared to the Normal CR group. Both body dissatisfaction and drive for thinness are closely related to dietary restraint in populations of women with eating disorders [5].

Weight Concern Score

Weight concern was assessed with a 5-item questionnaire [33]. The following five questions are asked of the participants (1) how much more or less do you feel you worry about your weight and body shape than other women your age?, (2) how afraid are you of gaining 3 pounds?, (3) how often have you dieted in the past?, (4) compared to other things in your life, how important is your weight to you?, and (5) do you ever feel fat? The weight concern score was used to describe dietary attitudes in the High CR group compared to the Normal CR group. Weight concern is also related to dietary restraint and is a corroborative measure of dietary restraint.
Fat Preference Questionnaire

The Fat Preference Questionnaire is a 22-itean questionnaire to assess fat preferences and actual intake of fat in foods [34]. Questions regarding food intake and food preference involve food examples taken from all four food groups. The Fat Preference Questionnaire was used to describe dietary attitudes in the High CR group compared to the Normal CR group.

Women with elevated dietary restraint typically avoid calorie dense food that are high in fat [35].

Weight Loss Strategy Questionnaire

The Weight Loss Strategy Questionnaire is a 24-iteam questionnaire used to measure healthy and unhealthy dieting behaviors [36]. Healthy dieting scores can range from 0 to 22 and high scores indicate a greater use of healthy dieting behaviors, e.g. reducing caloric intake, eliminating snacks, increasing exercise, increasing fruit and vegetable intake and decreasing fat intake. Unhealthy dieting scores can range from 0 to 18 and higher scores indicated greater use of unhealthy dieting behaviors, e.g. fasting, skipping meals, increasing cigarette use, diuretic use, and vomiting. Dieting is prevalent among young women and may be associated with both healthy and unhealthy eating attitudes [36]. The Weight Loss Strategy Questionnaire was used to describe both these healthy and unhealthy eating attitudes in the High CR group compared to the Normal CR group.

Power of Food Scale

The Power of Food Scale is a 15-iteam questionnaire used to measure the psychological impact of a food [37]. Appetite is assessed based on (1) food available, (2)
food present, and (3) food tasted. Higher scores represent higher appetite in the three food situations. The Power of Food Scale was used to describe eating attitudes in the High CR group compared to the Normal CR group.

High scores on the Power of Food Scale are correlated with elevated disinhibition and hunger (TFEQ) and with the emotional eating and external eating (DEBQ) [37].

**Beck Depression Inventory**

The Beck depression inventory is a 21-item questionnaire used to assess severity of current depressive symptoms [38]. Depressive scores range from 0 to 63 and higher scores indicate greater depression. The Beck depression inventory was used to describe current depressive symptoms in the High CR group compared to the Normal CR group. Typically, depression is not related to dietary restraint, however women with bulimia are more likely than the nonbulimics to have elevated depression and dietary restraint [39].

**Anthropometric Testing:**

Total body mass was measured to the nearest 0.1 kg on a physician’s scale (Seca, Model 770, Hamburg, Germany), and height was measured to the nearest 0.5 cm. Body mass index (BMI) was calculated as weight divided by height squared (kg/m²). Percentage body fat, fat mass, and lean body mass were determined by dual-energy X-ray absorptiometry (DXA, GE Lunar iDXA encore 2002 Software version 6.50.069). In our precision study, we scanned 13 women three times on the Lunar iDXA. The root-mean-square coefficient of variation values for the iDXA were 0.4%, 1.0%, and 0.9% for lean mass, fat mass and percent body fat, respectively.
Test Condition

Participants arrived at the clinical research center at ~1100h and were instructed not to consume food within 12 h prior to blood sampling and not to exercise or consume caffeine within 24 h prior to blood sampling. Compliance to these conditions was assessed prior to the protocol commencing. Testing was conducted on on days 2-11 of the menstrual cycle. In two of the women who were amenorrheic testing was conducted on an arbitrary day.

The test condition involved the consumption of a mixed composition standardized test meal, as described below. The catheter was inserted at ~30min (~1115h). Repeated blood samples were obtained at -15, 0, 10, 15, 20, 30, 60, 90, 120 and 180 min via an intravenous catheter (Figure 1), while the participants lay supine. The test meal was consumed between time 0 min and 10 min. Blood samples at -15 and 0 min were combined and considered the baseline (fasting) blood sample.

Figure 1. The test condition involved the consumption of a mix composition standardized test meal. Repeated blood samples were obtained at -15, 0, 10, 15, 20, 30, 60, 90, 120 and 180 min via an intravenous catheter, while the participants lay supine. The test meal was consumed before between time 0 min and 10 min.
Test Meal

The mixed composition test meal was a yogurt parfait. The energy content was relative to body weight, i.e., 5.0 kcal/kg. The parfait consisted of 64% carbohydrate, 22% fat, and 14% protein. The parfait was made with French vanilla low fat yogurt, sliced strawberries, granola, and crushed almonds. Subjects were instructed to consume the entire yogurt parfait meal in 10 min.

Visual Analogue Scales (VAS)

Subjective appetite sensations were assessed by VAS. VAS were given at -15, 0, 10, 15, 20, 30, 60, 90, 120 and 180 min. The motivation-to-eat VAS questionnaire (used to assess appetite) was composed of four questions or scales: 1) how strong is your desire to eat right now? (“NOT strong at all” to “extremely strong”), 2) how hungry do you feel right now? (“NOT hungry at all” to “extremely”), 3) how full do you feel right now? (“NOT Full at all” to “extremely full”) and 4) how much food do you think you can eat right now? (“nothing at all” to “a large amount “). Each VAS consisted of a 100 mm line anchored at the beginning and end by opposing statements. The subjects marked a single vertical mark on the line to indicate their feelings at the given moment. Scores were determined by measuring the distance (mm) from the left starting point of the line to the single vertical mark.

PYY\textsubscript{3-36} Measurements

Samples were collected into containers with ethylenediaminetetraacetic acid (EDTA), gently inverted and then aliquoted as quickly as possible (within 30 sec) into tubes that contained aprotinin (500 KIU). Samples were inverted, transferred into tubes that contained dipeptidyl peptidase-4 inhibitor (10uL per mL), and then centrifuged at
3000 rpm for 15 min at 4°C. The treated plasma was aliquoted into polyethylene storage tubes and stored frozen at −80°C until analysis. PYY\textsubscript{3-36} was measured using a radioimmunoassay (RIA) for total PYY (Linco Research, St. Charles, MO). The intra-assay and inter-assay coefficients of variation were 8.7 and 11.0%, respectively. The sensitivity of the assay was 20 pg/ml.

**Acylated Ghrelin Measurements**

Samples were collected into containers with EDTA, gently inverted, aliquoted, and then centrifuged at 3000 rpm for 15 min at 4°C. The plasma was aliquoted into polyethylene storage tubes that contained 1 N HCl, and Phenylmethylsulfonyl fluoride was added prior to the sample being stored at −80°C until analysis. Acylated ghrelin was measured using an RIA for active ghrelin (Linco Research, St. Charles, MO). The intra-assay and inter-assay coefficients of variation were 7.5 and 13.5%, respectively. The sensitivity of the assay was 7.8 pg/ml.

**Insulin Measurements**

Samples were allowed to clot for 30 min at room temperature (20–24°C) and then centrifuged at 3000 rpm for 15 min at 4°C. The serum was aliquoted into 2-mL polyethylene storage tubes and stored at −80°C until analysis. Insulin was analyzed using a chemiluminescence immunoassay analyzer (Immulite, Diagnostic Products Corporation, Los Angeles, CA) through immunometric assay. The intraassay and interassay coefficients of variation were 6.4% and 8.0%, respectively. Analytical sensitivity for the insulin assay was 2 μIU/mL.
Glucose Measurements

Samples were collected into containers with EDTA gently inverted and analyzed for glucose using a HemoCue Glucose System (Quest Diagnostics, Pittsburgh, PA) within 5 min of the blood draw.

Statistical Analysis

Data screening was conducted prior to statistical analysis in order to identify whether the data met the assumptions required by specific statistical techniques. Data screening involved outlier detection and examination of variable distributions within each of the two groups for normality. Since all distributions were normal, parametric analyses were utilized for inferential analyses. Repeated-measures ANOVA was used to determine whether changes in PYY3-36, active ghrelin, insulin, glucose concentrations, and VAS scores were significantly different following a meal in women with High CR compared to women with Normal CR. The area under the curve, mean, and peak concentrations following the meal were calculated for PYY3-36, active ghrelin, insulin, and glucose. For comparisons of area under the curve, mean, and peak concentrations between High CR and Normal CR, independent t-tests were employed. Simple linear regression, using p=0.05 for entry and p=0.010 to leave the model, was used to detect associations between variables of interest. All analyses were performed using SPSS software (version 18.0; SPSS, Inc., Chicago, IL). All data were reported as mean ± sem.

For PYY, sample size was based on the detection of a meaningful difference of 5 pmol/L and an SD of 23pmol/L following a meal in women with high disinhibition compared to women with normal disinhibition, based on a previously published report [2]. In order to achieve 80% power for the repeated measures analysis of PYY using a
0.05 level of significance, a sample size of 18 participants per group (total n= 36) was required.

For ghrelin, sample size was based on the detection of a meaningful difference of 100 pmol/l and an SD of 550 pmol/L following a meal in women with high dietary restraint compared to women with normal dietary cognitive restraint, based on a previously published report [40]. In order to achieve 80% power for the repeated measured analysis of ghrelin using a 0.05 level of significance, a sample size of 13 participants per group (total n= 26) was required.

RESULTS

Participant Characteristics

Descriptive demographic, body composition, menstrual cycle, and self reported physical activity is described in Table 1. The women in the study had a mean BMI of 23.4±0.5 kg/m², ranging from 18.8 to 31.9 kg/m². Seventy-nine percent of the participants studied were normal weight, 14% of the participants were overweight, and 7% of the population was considered obese. Body mass index in the High CR group ranged from 21.9 to 30.4 kg/m² and BMI in the Normal CR group ranged from 18.8 to 31.9 kg/m². Body weight (p=0.008), BMI (p=0.019), and lean body mass (p=0.023) were higher in the High CR group compared to the Normal CR group.
Table 1. Demographic characteristics, body composition, menstrual cycle, and self reported physical activity of the college-aged women with different eating behavior phenotypes.

<table>
<thead>
<tr>
<th></th>
<th>Normal CR (n=30)</th>
<th>High CR (n=13)</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td><strong>Demographic characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>21.0±0.3</td>
<td>20.1±0.3</td>
<td>0.075</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>164.3±1.1</td>
<td>165.6±1.7</td>
<td>0.539</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>61.1±1.5</td>
<td>69.1±2.8</td>
<td>0.008</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>22.7±0.6</td>
<td>25.1±0.6</td>
<td>0.019</td>
</tr>
<tr>
<td><strong>Body composition characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>29.6±1.4</td>
<td>32.4±1.6</td>
<td>0.253</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>18.4±1.2</td>
<td>22.6±2.0</td>
<td>0.067</td>
</tr>
<tr>
<td>Lean body mass (kg)</td>
<td>40.1±0.8</td>
<td>43.3±0.9</td>
<td>0.023</td>
</tr>
<tr>
<td><strong>Menstrual cycle characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age of menarche (yrs)</td>
<td>12.4±0.3</td>
<td>13.2±0.5</td>
<td>0.180</td>
</tr>
<tr>
<td>Number of menstrual cycles in the past 12 months</td>
<td>11.1±0.5</td>
<td>11.5±0.3</td>
<td>0.567</td>
</tr>
<tr>
<td><strong>Exercise history</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Physical activity (min/week)</td>
<td>366±51</td>
<td>395±64</td>
<td>0.746</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM

**Eating Behaviors**

Eating behaviors, measured by the TFEQ, DEBQ, EDI-2, and Weight Concern Questionnaire are presented in Table 2. By design, the High CR group had higher dietary restraint measured by both the TFEQ (p<0.001) and the DEBQ (p<0.001) compared to the Normal CR group. Additionally, the High CR group had higher flexible control (p<0.001) and rigid control (p<0.001) compared to the Normal CR group. Disinhibition measured by the TFEQ (p=0.042) was also higher in the High CR group compared to the Normal CR group. The High CR group also had a higher weight concern score (p<0.001) compared to the Normal CR group. The High CR group did not
like high fat food (p=0.050) compared to the Normal CR group and the High CR group
did not eat high fat food (p=0.001) as often as the Normal CR group. The High CR
group had more healthy (reducing caloric intake, eliminating snacks, increasing exercise,
increasing fruit and vegetable intake and decreasing fat intake, p=0.010) and unhealthy
(fasting, skipping meals, increasing cigarette use, diuretic use, and vomiting, p=0.031)
dieting behaviors compared to the Normal CR group.

Since baseline differences were demonstrated in BMI between the two groups and
since relationships between PYY and BMI have previously been demonstrated in the
literature [7, 22], we controlled for baseline differences in BMI for all of our analysis.
After controlling for group differences in BMI, the High CR group still exhibited higher
dietary restraint measured by both the TFEQ (p<0.001) and the DEBQ (p<0.001)
compared to the Normal CR group and the High CR group also had higher flexible
control (p<0.001) and rigid control (p<0.001) scores compared to the Normal CR group.
However, after controlling for group differences in BMI, differences in disinhibition were
no longer significant (p=0.107).
### Table 2. Eating behavior characteristics of the college-aged women with different eating behavior phenotypes.

<table>
<thead>
<tr>
<th></th>
<th>Normal CR (n=30)</th>
<th>High CR (n=13)</th>
<th>P value</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Three Factor Eating Questionnaire</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cognitive Restraint</td>
<td>7.4±0.4</td>
<td>14.1±0.3</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Disinhibition</td>
<td>4.8±0.5</td>
<td>6.8±0.8</td>
<td>0.042</td>
<td>0.107</td>
</tr>
<tr>
<td>Hunger</td>
<td>5.6±0.6</td>
<td>4.1±0.9</td>
<td>0.161</td>
<td>0.268</td>
</tr>
<tr>
<td>Flexible Control</td>
<td>2.4±0.2</td>
<td>4.8±0.2</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Rigid Control</td>
<td>2.8±0.3</td>
<td>5.2±0.2</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Dutch Eating Behavior Questionnaire</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cognitive Restraint</td>
<td>2.3±0.1</td>
<td>3.1±0.1</td>
<td>&lt;0.001</td>
<td>0.005</td>
</tr>
<tr>
<td>Emotional Disinhibition</td>
<td>2.0±0.1</td>
<td>2.2±0.2</td>
<td>0.308</td>
<td>0.365</td>
</tr>
<tr>
<td>External Disinhibition</td>
<td>3.0±0.1</td>
<td>2.8±0.1</td>
<td>0.287</td>
<td>0.242</td>
</tr>
<tr>
<td>Diffuse Emotional Disinhibition</td>
<td>2.5±0.2</td>
<td>2.5±0.2</td>
<td>0.806</td>
<td>0.775</td>
</tr>
<tr>
<td>Specific Emotional Disinhibition</td>
<td>1.8±0.2</td>
<td>2.1±0.2</td>
<td>0.218</td>
<td>0.287</td>
</tr>
<tr>
<td>Overall Disinhibition</td>
<td>2.4±0.1</td>
<td>2.5±0.2</td>
<td>0.762</td>
<td>0.857</td>
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<tr>
<td><strong>Eating Disorder Inventory</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Dissatisfaction</td>
<td>5.3±1.4</td>
<td>10.0±1.6</td>
<td>0.052</td>
<td>0.467</td>
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<tr>
<td>Drive for Thinness</td>
<td>1.9±0.7</td>
<td>3.4±1.1</td>
<td>0.223</td>
<td>0.925</td>
</tr>
<tr>
<td><strong>Weight Concern Questionnaire</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight Concern Score</td>
<td>1.1±0.1</td>
<td>1.8±0.1</td>
<td>0.003</td>
<td>0.030</td>
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<tr>
<td><strong>Fat Preference Questionnaire</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Likes High Fat Foods Better</td>
<td>1.03±0.04</td>
<td>0.88±0.07</td>
<td>0.050</td>
<td>0.029</td>
</tr>
<tr>
<td>Eats More of High Fat Foods</td>
<td>0.76±0.03</td>
<td>0.54±0.06</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Weight Loss Strategy Questionnaire</strong></td>
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<tr>
<td>Healthy Dieting Behaviors</td>
<td>28.8±1.7</td>
<td>36.6±1.9</td>
<td>0.010</td>
<td>0.070</td>
</tr>
<tr>
<td>Unhealthy Dieting Behaviors</td>
<td>9.5±0.2</td>
<td>10.2±0.3</td>
<td>0.031</td>
<td>0.115</td>
</tr>
<tr>
<td><strong>Power of Food Scale</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Power of Food Available</td>
<td>10.8±0.8</td>
<td>10.1±1.3</td>
<td>0.628</td>
<td>0.644</td>
</tr>
<tr>
<td>Power of Food Present</td>
<td>9.6±0.7</td>
<td>8.6±1.0</td>
<td>0.455</td>
<td>0.480</td>
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<tr>
<td>Power of Food Tasted</td>
<td>11.4±0.6</td>
<td>10.2±1.0</td>
<td>0.298</td>
<td>0.337</td>
</tr>
<tr>
<td><strong>Beck Depression Inventory</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beck Depression Score</td>
<td>3.3±0.7</td>
<td>3.4±0.8</td>
<td>0.941</td>
<td>0.789</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM  *Controlled for body mass index
Plasma Concentrations of PYY3-36, Active Ghrelin, Insulin, and Glucose

Fasting and meal related response patterns of PYY3-36, active ghrelin, insulin, and glucose concentrations are presented in Table 3. Before (-15 and 0 min) and after (10, 15, 20, 30, 60, 90, 120 and 180 min) the consumption of a mixed composition meal, mean PYY3-36 concentrations (p=0.042), peak PYY3-36 concentrations (p=0.047) and PYY3-36 area under the curve (p=0.035) were lower in the High CR group compared to the Normal CR group, after controlling for BMI. However, fasting (-15 and 0 min combined) PYY3-36 concentrations were not different (p=0.124) in the High CR group compared to the Normal CR group, after controlling for BMI.
Table 3. Peptide YY3-36, active ghrelin, insulin, and glucose meal responses of the college-aged women different eating behavior phenotypes.

<table>
<thead>
<tr>
<th></th>
<th>Normal CR (n=30)</th>
<th>High CR (n=13)</th>
<th>P value</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PYY3-36 Concentrations</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting PYY3-36 (pg/ml)</td>
<td>36.9±3.5</td>
<td>28.7±4.9</td>
<td>0.189</td>
<td>0.124</td>
</tr>
<tr>
<td>Mean PYY3-36 (pg/ml)</td>
<td>41.0±3.1</td>
<td>32.1±3.6</td>
<td>0.103</td>
<td><strong>0.042</strong></td>
</tr>
<tr>
<td>Peak PYY3-36 (pg/ml)</td>
<td>58.8±4.7</td>
<td>49.3±5.2</td>
<td>0.235</td>
<td><strong>0.047</strong></td>
</tr>
<tr>
<td>PYY3-36 AUC</td>
<td>7709±612</td>
<td>6022±639</td>
<td>0.107</td>
<td><strong>0.035</strong></td>
</tr>
</tbody>
</table>

| **Active Ghrelin Concentrations** |                  |                |         |         |
| Fasting active Ghrelin (pg/ml)  | 90.6±6.9         | 87.4±10.7      | 0.805   | 0.925   |
| Mean active Ghrelin (pg/ml)     | 90.7±5.7         | 80.7±10.3      | 0.371   | 0.625   |
| Peak active Ghrelin (pg/ml)     | 126.4±8.3        | 105.0±12.9     | 0.165   | 0.365   |
| Active ghrelin AUC              | 16024±1073       | 13811±1887     | 0.286   | 0.539   |

| **Insulin Concentrations** |                  |                |         |         |
| Fasting insulin (uIU/ml)      | 2.4±0.1          | 2.4±0.2        | 0.943   | 0.824   |
| Mean insulin (uIU/ml)         | 12.2±1.1         | 12.8±1.5       | 0.759   | 0.808   |
| Peak insulin (uIU/ml)         | 32.9±3.0         | 33.9±4.2       | 0.827   | 0.598   |
| Insulin AUC                   | 2640±206         | 2926±353       | 0.467   | 0.813   |

| **Glucose Concentrations**   |                  |                |         |         |
| Fasting glucose (mg/dl)       | 95.5±1.7         | 97.3±2.7       | 0.583   | 0.570   |
| Mean glucose (mg/dl)          | 106.8±1.4        | 104.1±1.6      | 0.257   | 0.313   |
| Peak glucose (mg/dl)          | 133.8±2.4        | 126.5±3.1      | 0.091   | 0.112   |
| Glucose AUC                   | 21135±268        | 20482±299      | 0.157   | 0.223   |

Values are the mean ± SEM. PYY=Peptide YY. AUC = Area under the curve. *Controlled for body mass index

PYY3-36 concentrations before and after the test meal are presented in Figure 2A. There was no significant (p=0.353) time X group interaction. There was also no group effect (p=0.103), but there was a linear effect of time (p=0.013), indicating that PYY3-36 increased following the intake of the meal. However, after controlling for BMI differences, there was a significant group effect (p=0.043), indicating that the High CR group had lower circulating PYY3-36 concentrations compared to the Normal CR group after the test meal at time 15 min (p=0.043), 90 min (p=0.025), and 180 min (p=0.009). To determine if there were group differences after the meal, we analyzed the data from time 10 to 180 min alone. There was no significant time X group interaction (p=0.302)
and no group effect (p=0.103), but there was a linear effect of time (p=0.038), indicating that PYY$_{3-36}$ increased following the intake of the meal. Additionally, after controlling for BMI, there was a group effect (p=0.044) during this time period. To determine if the groups differed in their responses at different times during the post meal period, the data were divided from time 0 to 60 min, 60 to 120 min, 120 to 180 min with the time period of 120-180 min considered the most important for PYY$_{3-36}$ and considered the most important 10-20 min for ghrelin. For the time period of 0 to 60 min there was no significant time X group interaction (p=0.526), no group effect (p=0.199), or no time effect (p=0.468). From the time period of 60 to 120 min there was a time X group interaction (p=0.044), however, the significant interaction was lost when controlling for BMI (p=0.178). From 120 to 180 min there was no significant time X group interaction (p=0.332), no group effect (p=0.076), or no time effect (p=0.748). However, there was a group effect when controlling for BMI (p=0.015), indicating that the High CR group had lower circulating PYY$_{3-36}$ concentrations from 120 to 180 min after the test meal.

Active ghrelin concentrations before and after the test meal are presented in Figure 2B. There was no significant (p=0.900) time X group interaction. There was also no group effect (p=0.363). However, there was a quadratic effect of time (p<0.001), indicating that active ghrelin concentrations decreased following the meal and then started to increase approximately 60 min following the meal. No differences were observed after controlling for BMI.
Figure 2.
Figure 2. (a) PYY$_{3-36}$ concentrations over time following a test meal, after controlling for BMI. The test meal was consumed with 10 min (shown in grey). There was no significant (p=0.353) time X group interaction. There was also no group effect (p=0.103), but there was a linear effect of time (p=0.013). However, after controlling for BMI differences, there was a significant group effect (p=0.043). Follow-up analysis, also controlling for BMI, demonstrated that PYY$_{3-36}$ concentrations were lower in the High CR group compared to the Normal CR group after the test meal a time 15min (p=0.043), 90 min (p=0.025), and 180min (p=0.009), *p<0.05. (b) Active Ghrelin concentrations over time following a test meal. The test meal was consumed with 10 min (shown in grey). There was no significant (p=0.900) time X group interaction. There was also no group effect (p=0.363). However, there was a quadratic effect of time (p<0.001). No differences occurred after controlling for BMI.
Glucose concentrations before and after the test meal are presented in Figure 3A. There was no significant (p=0.271) time X group interaction and no group effect (p=0.181). However, there was a quadratic effect of time (p<0.001), indicating that glucose concentrations increased after the meal, followed by a decrease approximately 30 minutes after the meal. No differences were observed after controlling for BMI.

Insulin concentrations before and after the test meal are presented in Figure 3B. There was no significant (p=0.808) time X group interaction. There was also no group effect (p=0.751). However, there was a quadratic effect of time (p<0.001) indicating that insulin concentrations increased after the meal, followed by a decrease approximately 30 minutes after the meal. No differences were observed after controlling for BMI.
Figure 3.
Visual Analogue Scales

Hunger, fullness, desire to eat, and prospective food consumption scores before and after a test meal are presented in Figure 4. There was no significant (p>0.05) time X group interaction or group effect (p>0.05) on any VAS. However, there was a quadratic effect of time (p<0.001) on all VAS. Fullness increased after the meal followed by a gradual decrease during the 3 hour postprandial period, while hunger, desire to eat, and prospective food consumption decreased after the meal followed by a gradual increase during the 3 hour postprandial period. No differences occurred after controlling for BMI.

Figure 3. (a) Glucose concentrations over time following a test meal. The test meal was consumed with 10 min (shown in grey). There was no significant (p=0.271) time X group interaction. There was also no group effect (p=0.181). However, there was a quadratic effect of time (p<0.001). No differences occurred after controlling for BMI. (b) Insulin concentrations over time following a test meal. The test meal was consumed with 10 min (shown in grey). There was no significant (p=0.808) time X group interaction. There was also no group effect (p=0.751). However, there was a quadratic effect of time (p<0.001). No differences occurred after controlling for BMI.
Figure 4.
Correlations

Pearson correlations between subscales of the Three Factor Eating Questionnaire (dietary cognitive restraint, disinhibition, and hunger), body composition, and appetite signals are presented in Table 4. Dietary restraint was positively (p<0.05) correlated with body mass, BMI, lean body mass and fat free mass. Pearson correlations between body mass and body mass index and appetite signals are presented in Table 5. Body mass was negatively (p<0.05) correlated with ghrelin AUC.
Table 4. Pearson correlations between subscales of the Three Factor Eating Questionnaire (dietary cognitive restraint, disinhibition, and hunger), body composition, and appetite signals.

<table>
<thead>
<tr>
<th></th>
<th>Dietary Cognitive Restraint</th>
<th>Disinhibition</th>
<th>Hunger</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R-value p-value</td>
<td>R-value p-value</td>
<td>R-value p-value</td>
</tr>
<tr>
<td><strong>Body Composition</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Mass (kg)</td>
<td>0.463 0.002**</td>
<td>0.274 0.075</td>
<td>-0.116 0.457</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>0.397 0.008**</td>
<td>0.230 0.137</td>
<td>-0.157 0.314</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>0.340 0.026*</td>
<td>0.175 0.263</td>
<td>-0.222 0.152</td>
</tr>
<tr>
<td>Lean Body Mass (kg)</td>
<td>0.415 0.006**</td>
<td>0.259 0.093</td>
<td>0.124 0.427</td>
</tr>
<tr>
<td><strong>Appetite Hormones</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peptide YY AUC</td>
<td>-0.117 0.454</td>
<td>0.085 0.590</td>
<td>-0.056 0.721</td>
</tr>
<tr>
<td>PYY3-36 180min after meal (ng/dl)</td>
<td>-0.196 0.208</td>
<td>0.035 0.823</td>
<td>0.042 0.787</td>
</tr>
<tr>
<td>Ghrelin AUC</td>
<td>-0.196 0.207</td>
<td>-0.156 0.318</td>
<td>-0.003 0.985</td>
</tr>
<tr>
<td>Ghrelin 180min after meal (ng/dl)</td>
<td>-0.174 0.346</td>
<td>-0.011 0.943</td>
<td>0.087 0.581</td>
</tr>
<tr>
<td>Insulin AUC</td>
<td>0.116 0.457</td>
<td>0.180 0.247</td>
<td>0.012 0.940</td>
</tr>
<tr>
<td>Insulin Peak (uIU/ml)</td>
<td>0.072 0.648</td>
<td>0.128 0.412</td>
<td>0.034 0.831</td>
</tr>
<tr>
<td>Glucose AUC</td>
<td>-0.100 0.525</td>
<td>0.135 0.388</td>
<td><strong>0.454 0.002</strong> **</td>
</tr>
<tr>
<td>Glucose Peak (mg/dl)</td>
<td>-0.115 0.462</td>
<td>-0.012 0.942</td>
<td><strong>0.337 0.027</strong> *</td>
</tr>
</tbody>
</table>

**Significant correlations (p<0.01)
*Significant correlations (p<0.05)

AUC = area under the cure
**Table 5.** Pearson correlations between body mass and body mass index and appetite signals.

<table>
<thead>
<tr>
<th>Appetite Hormones</th>
<th>Body Mass R-value</th>
<th>p-value</th>
<th>Body Mass Index R-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide YY AUC</td>
<td>-</td>
<td>0.848</td>
<td>0.144</td>
<td>0.356</td>
</tr>
<tr>
<td>PYY&lt;sub&gt;3-36&lt;/sub&gt; 180 min after meal (ng/dl)</td>
<td>-</td>
<td>0.982</td>
<td>0.214</td>
<td>0.169</td>
</tr>
<tr>
<td>Ghrelin AUC</td>
<td>-</td>
<td>0.046*</td>
<td>-2.17</td>
<td>0.162</td>
</tr>
<tr>
<td>Ghrelin 180 min after meal (ng/dl)</td>
<td>0.249</td>
<td>0.108</td>
<td>-0.168</td>
<td>0.282</td>
</tr>
<tr>
<td>Insulin AUC</td>
<td>0.203</td>
<td>0.191</td>
<td>0.223</td>
<td>0.151</td>
</tr>
<tr>
<td>Insulin Peak (uIU/ml)</td>
<td>0.216</td>
<td>0.165</td>
<td>0.304</td>
<td>0.047*</td>
</tr>
<tr>
<td>Glucose AUC</td>
<td>-</td>
<td>0.196</td>
<td>-0.115</td>
<td>0.463</td>
</tr>
<tr>
<td>Glucose Peak (mg/dl)</td>
<td>0.201</td>
<td>-0.375</td>
<td>-0.081</td>
<td>0.607</td>
</tr>
</tbody>
</table>

**Significant correlations (p<0.01)**

AUC = area under the cure

**Regression Analyses**

To further explore the relationships between PYY and eating behavior, simple linear regression analyses demonstrated that flexible control and rigid control predict 30.5% of the variability in PYY AUC (Table 6).

To further explore the dietary restraint and gastrointestinal hormones, we demonstrated, using simple linear regression, that BMI and PYY<sub>3-36</sub> 180 min after the test meal predicted 20.2% of the variability in dietary restraint (Table 7).
Table 6. Results of regression analysis for predictors of Peptide YY$_{3-36}$ area under the curve following a test meal in young college-aged women.

<table>
<thead>
<tr>
<th></th>
<th>β value</th>
<th>p value</th>
<th>Variability Explained by Variable</th>
<th>Cumulative Variability Explained by Model</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PYY AUC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flexible Control*</td>
<td>-1633.4</td>
<td>0.011</td>
<td>12.6%</td>
<td>12.6%</td>
</tr>
<tr>
<td>Rigid Control*</td>
<td>1074.6</td>
<td>&lt;0.001</td>
<td>17.9%</td>
<td>30.5%</td>
</tr>
</tbody>
</table>

PYY=Peptide YY. AUC = Area under the curve.
*Rigid and Flexible are subscales of the Three Factor Eating Questionnaire [27]. Rigid control scores indicate an ‘all or nothing’ approach to eating and dieting, while Flexible control indicates a more moderate approach to eating and dieting [27, 28].

Table 7. Results of regression analysis for predictors of dietary cognitive restraint following a test meal in young college-aged women.

<table>
<thead>
<tr>
<th></th>
<th>β value</th>
<th>p value</th>
<th>Variability Explained by Variable</th>
<th>Cumulative Variability Explained by Model</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dietary Cognitive Restraint</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Mass Index</td>
<td>0.582</td>
<td>0.008</td>
<td>13.7%</td>
<td>13.7%</td>
</tr>
<tr>
<td>PYY$_{3-36}$ 180min after the test meal</td>
<td>-0.045</td>
<td>0.004</td>
<td>6.5%</td>
<td>20.2%</td>
</tr>
</tbody>
</table>

PYY=Peptide YY
DISCUSSION

The most important findings from this study indicate that women with high dietary restraint have low PYY\textsubscript{3-36} concentrations several hours after a meal and this may be indicative of a blunted appetite reduction after a meal and, over time, lead to an increased risk of weight gain. This finding is in contrast to our hypothesis, as PYY\textsubscript{3-36} concentrations were suppressed in the women in the High CR group compared to the Normal CR group after the test meal. As such, the results of this study suggest that the women with the eating behavior phenotype of elevated dietary restraint may be compensating for reduced physiological signalling of PYY\textsubscript{3-36} circulating concentrations after a meal by consciously restraining food intake. On the other hand, no differences were observed in active ghrelin concentrations, insulin, glucose, hunger, fullness, and desire to eat after the test meal between the different eating behavior phenotypes indicating that dietary restraint has no impact on measures of appetite and on other hormones that are altered with food intake.

In this study we demonstrated that flexible control and rigid control predicted 30.5\% of the variability of PYY AUC. In this regression model, flexible control had a negative relationship with PYY AUC, indicating that low PYY AUC was predicted by high flexible control. This finding can be interpreted to mean that successful dietary restraint measured by flexible control is related to low circulating PYY concentrations and that successful dietary restraint may be necessary to compensate for reduced PYY signaling to avoid weight gain. In the literature, high flexible control is associated with low disinhibition and less binge eating in obese and non-obese women [27, 30]. Rigid
control has a positive relationship with PYY AUC in our regression model, indicating that low PYY AUC is predicted by low rigid control. Rigid control represents an 'all or none' approach to dieting, associated with high disinhibition, high BMI and more frequent binge eating [27, 29, 30]. The regression modeling in the current study supports the notion that successful dietary restraint related to flexible control, and not rigid control, is related to reduced physiological signalling of PYY3-36 circulating concentrations after a meal. Elevated flexible control may be a behavioral mechanism compensating for the reduced physiological signalling of PYY3-36 circulating concentrations after a meal and may be an approach to weight control and to the avoidance of weight gain.

Interestingly, in the current study BMI and PYY3-36 180 min after the test meal predict 20.2% of the variability in dietary restraint. Elevated dietary restraint around 3 hours following a meal may have important implications regarding the next meal or snack. In an epidemiological study, Zizza et al [23] demonstrated that snacking prevalence and energy intake from snacks has increased in the United States, and we speculate that individual differences in meal frequency associated by circulating concentrations of PYY could have effects on energy intake. Elevated dietary restraint may be a behavioral mechanism compensating for the reduced physiological signalling of PYY3-36 around the time of a next meal or snack and may be an important approach to weight control.

In the current study, PYY3-36 concentrations were suppressed in our participants in the High CR group compared to Normal CR; we speculate that these women may have elevated dietary restraint to compensate for suppressed PYY3-36 circulating
concentrations after a meal signaling less appetite suppression as PYY3-36 has been identified as a satiety factor. Results of other studies have provided evidence that women with the specific eating behavior phenotype of high dietary restraint have altered metabolic and endocrine homeostasis [1, 14, 41-43]. In 1990, Pirke et al. [41] investigated normal weight women with high dietary restraint and demonstrated that they had suppressed insulin concentrations at night and suppressed norepinephrine concentrations following a standardized test meal. Pirke et al. [41] proposed that high dietary restraint may be necessary for the women to remain weight stable over time since suppressed energy expenditure is likely related to suppressed insulin and norepinephrine concentrations. Pirke et al. [41] further argued that since the women were weight stable and failed to exhibit other metabolic alterations suggestive of an energy deficiency, the alterations in insulin and norepinephrine were not a result of dieting and weight loss. To this end, Tuschl et al. [42] reported that women with high dietary restraint had suppressed total daily energy expenditure, measured by doubly labelled water, and suppressed dietary intake, measured by food logs, and suggested that high restraint was the result of suppressed energy expenditure. Similar to Pirke et al. [41] we did not see alterations in insulin following a meal in women with high dietary restraint compared to normal dietary restraint. Our study may extend the findings of Pirke et al. [41] by including PYY in the list of observed biological responses associated with distinct eating behavioral phenotypes in young college-aged women.

Elevated metabolism is associated with elevated PYY after a meal [44, 45]. Doucett et al. [44] measured PYY for 3 hours following a standardized test meal of 575 kcals and demonstrated that PYY is associated with the thermic effect of a meal and
postprandial energy expenditure in healthy women. In the current study, we demonstrated that women with high dietary restraint have suppressed PYY, and we speculate that the women in our study with high dietary restraint may have suppressed energy expenditure following that meal. This again supports the notion that women with the eating behavior phenotype of high dietary restraint have an increased risk of weight gain over time and that high dietary restraint is necessary to compensate for suppressed energy expenditure \[42\] and circulating PYY$_{3-36}$ concentrations after a meal to avoid weight gain.

Relationships between PYY feeding dynamics and eating behavior phenotypes have only recently been explored in normal weight populations \[2\]. Recently, Martins et al. \[2\] demonstrated that high dietary restraint did not affect fasting or postprandial total PYY concentrations; rather elevated disinhibition was associated with suppressed circulating PYY concentrations following a meal in normal weight men and women. One notable difference between the current study and the study by Martins et al. \[2\] was that men were included, while in the current study only women were investigated indicating that men and women may have different feeding dynamics and eating behavior phenotypes. Additionally, the study by Martins et al. \[2\] was a secondary analysis and not the primary focus of the design of the study.

To date, this is the first study to evaluate the active ghrelin response patterns after a test meal and to assess if ghrelin concentrations after a test meal discriminate between women with high dietary restraint and normal dietary cognitive restraint. In this study, we demonstrated that active ghrelin concentrations were not different between women with the distinct eating behavior phenotypes of high and normal dietary cognitive
restraint. St-Pierre et al. [46] also demonstrated no relationship between dietary restraint and fasting total ghrelin concentrations in young healthy women. However, Schur et al. [1] previously demonstrated in non obese men and women that fasting ghrelin concentrations were elevated in the high and intermediate dietary restraint groups compared to the low dietary restraint group. However, Schur et al. [1], used a modified TFEQ and high dietary restraint was defined as a cognitive restraint score greater than 4 (in the current study high dietary restraint was a score greater than 14), indicating that differences in study design may account for differences in the ghrelin findings. Importantly, many studies have also confirmed that ghrelin is tightly linked to energy balance [47-49] such that elevated circulating ghrelin concentrations are related to a negative energy balance. The findings in the current study suggest that the women with high dietary restraint do not exhibit signs of energy deficiency.

In the current study we corroborated the measures of dietary restraint measured by the TFEQ by several other measures. As expected, we demonstrated that the women with high dietary restraint do not eat high fat food and demonstrated both healthy and unhealthy eating patterns compared to women with normal dietary restraint. Importantly, the women with elevated dietary restraint did not demonstrate elevated drive for thinness or depression. This is important since elevated drive for thinness is indicative of disordered eating or anorexia nervosa [5] and elevated depression in combination with elevated dietary restraint could be indicative of bulimia [39].

The major limitation of this study is inherent in the cross-sectional design, and therefore we cannot determine if the specific eating behavior phenotype of high dietary restraint is causing suppressed PYY$_{3-36}$ concentrations or if suppressed PYY$_{3-36}$
concentrations are causing the eating behavior phenotype of high dietary cognitive restraint. However, we would not be able to infer causality even if we had completed a prospective study. There are many gut peptides and the physiological systems to control appetite that are highly redundant. The groups differed in very few ways other than in restraint score. However, the results from the current study cannot necessarily be extrapolated to other populations. The strengths of the current study include that active ghrelin and PYY\textsubscript{3-36} were measured, as opposed to total ghrelin and total PYY. The two forms of circulating PYY include PYY\textsubscript{1-36} and PYY\textsubscript{3-36}. PYY\textsubscript{3-36} is the major form of circulating PYY after a meal, while PYY\textsubscript{1-36} is the main form of circulating PYY during a fasted state [50]. PYY\textsubscript{3-36} is highly selective for the Y2 receptor and acts as an agonist for the Y2 receptor [51]. The ability of PYY\textsubscript{3-36} to bind to the Y2 receptor indicates the key pivotal role of this peptide in body weight regulation. Hypothalamic Y2 receptors are involved in both food intake and body weight regulation at the level of the hypothalamus [52]. To this end, the two forms of circulating ghrelin are acylated ghrelin and des-acylated ghrelin circulate in the blood, and in the current study we measured acylated ghrelin, the biologically active form of the hormone [53].

Future studies need to investigate PYY and dietary restraint in many different populations of women, i.e. normal weight women, obese women, and women with anorexia nervosa, to investigate if the interactions between PYY and dietary restraint differ in different populations. Additionally, future studies need to investigate PYY and dietary restraint longitudinally to examine the response of these factors impacting food intake over time, and if these changes are associated with changes in body weight. Lastly, future studies need to investigate if changes in BMI over time are more strongly
predicted by eating phenotypes or by gastrointestinal peptides like PYY. The more
eresearchers can understand with regards to the relationship between eating phenotypes
and appetite physiology the better we will be able to understand eating behaviors in
humans.

In conclusion, PYY$_{3-36}$ concentrations following a meal were different in the
women with different eating behavior phenotypes; PYY$_{3-36}$ concentrations were
suppressed in the High CR group compared to the Normal CR group. We speculate that
the specific eating behavior phenotype of elevated dietary restraint in college-aged
women may be a compensatory mechanism as a result of suppressed PYY$_{3-36}$ circulating
concentrations after a meal, indicating that suppressed PYY$_{3-36}$ may put the women at
risk for weight gain and cognitively restricting food intake may be an important
compensatory mechanism to avoid weight gain.
REFERENCES


CHAPTER VI


ABSTRACT

Weight gain in women with anorexia nervosa improves circulating concentrations of peptide YY (PYY) and ghrelin; however, no studies have investigated the effects of the reversal of low energy availability on PYY or ghrelin concentrations during the recovery of menses in women with exercise-associated menstrual cycle disturbances (EAMD). The purpose of this study was to determine if ghrelin and PYY are associated with the recovery of menses in women with EAMD during an intervention of increased caloric intake. We hypothesized that recovery of menses in women with EAMD will be associated with a decrease in fasting ghrelin and fasting PYY concentrations compared to women with EAMD who do not resume menses during 6 months of an increased caloric intake intervention. We speculate that decreases in circulating ghrelin and PYY concentrations will be mediated by favorable changes in body weight as a result of improvements in energy status, as indicated by an increase in total triiodothyronine (TT3). The current study includes data from 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 or a control group at the beginning of the intervention and after a baseline period of study. For the purposes of the current sub-study, the exercising women with oligomenorrhea and amenorrhea were re-categorized into one of two groups regardless of their initial group
assignment: 1) those who experienced menstrual recovery between months one and six of the intervention (EAMD-R; n = 15), and 2) those who did not resume menses within 6 months of the intervention (EAMD-NR; n = 9). At baseline the EAMD-R and EAMD-NR groups were similar with respect to age (p=0.288), height (p=0.561), weight (p=0.320), and BMI (p=0.133). There was no difference between the EAMD-R and EAMD-NR groups with respect to baseline ghrelin concentrations (p=0.245), PYY concentrations (p=0.164), and TT3 concentrations (p=0.899). Fifteen out of 24 women with EAMD experienced recovery of menses. EAMD-R group experienced menstrual recovery 64.9±13.2 days after the baseline period of the study. The change in body weight (p=0.008) and lean body mass (p=0.011) over 6 months was significantly different in the EAMD-R group compared to the EAMD-NR group, while there was no difference in the change in fat mass (p=0.313). Baseline, Intervention week 5, 9, and 21 time points demonstrated a significant time by experimental group interaction for fasting total ghrelin concentrations (p<0.001), indicating decreases in circulating ghrelin in the EAMD-R group. Baseline, Intervention week 5, 9, and 21 time points did not demonstrate a significant time by experimental group interaction for fasting total PYY concentrations (p=0.173), time effect (p=0.857), or group effect (p=0.656) in the EAMD-R group. There was a significant correlation between change in ghrelin and body weight (r=-0.521, p=0.013), and a significant correlation between change in ghrelin and BMI (r=-0.517, p=0.016). There was a significant correlation between change in mean urinary estrone -1-glucuronide (E1G ) and ghrelin (r=-0.539, p=0.010), and a significant correlation between change in mean E1G and body weight (r=0.616, p=0.002). In the current 6 month longitudinal study we demonstrated that circulating ghrelin
concentrations were decreased in women who experienced menstrual recovery compared
to the women who did not resume menses, indicating that elevated ghrelin may be
mechanistically involved in regulating reproduction in women with EAMD.

**INTRODUCTION**

Exercise-related menstrual cycle disturbances are often observed in women who
participate in physical activity ranging from the recreational to the competitive level [1, 2]. Severe exercise-associated menstrual cycle disturbances (EAMD), including
oligomenorrhea and “functional hypothalamic amenorrhea” (FHA), represent the most extreme presentation of menstrual irregularities [1, 3]. Low energy availability has been shown to be causally related to exercise associated menstrual disturbances [4, 5] and to impact reproductive function [6-9]. Energy availability has been operationally defined by one investigator [6, 10] as the difference between daily energy intake and energy expended during purposeful exercise adjusted for lean body mass. Short term manipulations of this expression of energy availability have demonstrated that low energy availability is associated with a decrease in luteinizing hormone (LH) pulse frequency [6-9], indicating a direct effect of low energy availability on reproductive function.

Alterations in metabolic and endocrine homeostasis associated with low energy availability in exercising women with FHA include: suppressed resting energy expenditure [11-13], reduced concentrations of total triiodothyronine (TT3) [11, 12, 14], leptin [15, 16], insulin-like growth factor-1 (IGF-1) [16], and insulin [13], and elevated concentrations of growth hormone (GH) [17], cortisol [2, 16, 18], ghrelin [14, 19, 20],
and peptide YY (PYY) [21, 22]. Some of these metabolic hormones may be involved mechanistically in the suppression of LH pulsatility and reproductive function. Moreover, these metabolic adjustments to low energy availability are associated with serious clinical conditions extending beyond suppressed reproductive function to include compromised bone health [23-25], and endothelial dysfunction [26-28]. As such, it becomes important to understand the factors involved in the reversal of low energy availability and the recovery of reproductive function to improve clinical outcomes.

To date, only a few metabolic hormones have been examined during the recovery of menses in exercising monkeys [4, 5] and exercising women [29] with amenorrhea. In a monkey model, Williams et al. [4, 5] demonstrated that amenorrhea, caused by an increase in exercise energy expenditure, can be reversed by increasing energy availability via food intake without a commensurate change in exercise expenditure. In these monkeys, an increase in circulating concentrations of TT3 were associated with the resumption of menses [4]. In a case study, Dueck et al. [29] demonstrated that increasing caloric intake and decreasing exercise training by one session per week improved metabolic signals, i.e., decreased cortisol concentrations. Although the athlete in the case study did not resume menses during the 15 week intervention period, an additional self-initiated three months of the study protocol reportedly led to the resumption of menses [29]. To this end, in a small study of four exercising women with amenorrhea, increased caloric intake by providing an additional 360 kcals/day, in the form of a nutritional supplement, and decreased training from 7 days/week to 6 days/week, demonstrated recovery of menses [30].
Improving energy status and increasing body weight has been demonstrated to be associated with resumption of menses in women with anorexia nervosa [31-33]. Additionally, weight gain in anorexia nervosa is associated with decreases in both PYY and ghrelin concentrations [34] and we speculate that the changes in one or both of these gastrointestinal hormones may be associated with the resumption of menses in exercising women with severe menstrual disturbances.

There is evidence to suggest that elevated concentrations of PYY and ghrelin may suppress reproductive function in women with FHA through central actions on hypothalamic neurons [35-41]. Studies using a rat model have suggested that elevations in ghrelin [39, 42] and PYY [41] concentrations can cause alterations in the secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus or LH from the anterior pituitary. While weight gain in women anorexia nervosa improves circulating concentrations of PYY and ghrelin [34, 43, 44], no studies to date in humans have been published that report on the effects of the reversal of low energy availability on PYY or ghrelin concentrations during the resumption of menses in exercising women with EAMD.

The purpose of this study was to determine if ghrelin and PYY are associated with the recovery of menses in women with EAMD during an intervention of increased caloric intake. We hypothesized that recovery of menses in women with EAMD will be associated with a decrease in fasting ghrelin and fasting PYY concentrations compared to women with EAMD who do not resume menses during 6 months of an intervention designed to increase caloric intake. We speculate that decreases in circulating ghrelin
and PYY concentrations will be mediated by favorable changes in body weight as a result of improvements in energy status, as indicated by an increase in TT₃.

PARTICIPANTS AND METHODS

Study Design

This sub-study was part of a randomized control trial that was designed to determine the effects of a 12-month intervention of increased caloric intake on indices of bone health and menstrual status in premenopausal women who suffer from severe EAMD, including oligomenorrhea (long and inconsistent menstrual cycles of 36-90 days) and FHA (the absence of menses for >90 days). The study was conducted at two sites, the University of Toronto and 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 experimental design is illustrated in Figure 1. The current study includes data from all women with severe EAMD who completed 6 months of the intervention designed to increase caloric intake 20-30% above baseline energy requirements. Participants were randomly assigned to a treatment group or a control group at the beginning of the intervention and after a baseline period of study. For the purposes of the current sub-study, the exercising women with oligomenorrhea and amenorrhea were re-categorized into one of two groups regardless of their initial group assignment: 1) those who experienced menstrual recovery between months one and six of the intervention (EAMD-R; n = 15), and 2) those who did not resume menses within 6
months of the intervention (EAMD-NR; n = 9). Of the 15 women assigned to the EAMD-R group, 9 were originally in the EAMD group who received an energy prescription of increased caloric intake (EAMD+Calories; n=12) and 6 were in the EAMD control group who maintained their baseline energy intake (EAMD Controls; n=12). Of the 9 women assigned to the EAMD-NR group, 3 were originally in the EAMD+Calories group and 6 were in the EAMD control group. The successful recovery of menses in women with exercise associated FHA at baseline was primarily defined as the first occurrence of menstrual bleeding that occurred during the intervention, and is detailed below. For additional analyses of the recovery of reproductive function, participants were assigned to groups based on several other indices of recovery of reproductive function, as detailed below (see Classification of Intervention Menstrual Status).

Repeated measures of dietary intake, body weight (kg), body composition (kg FFM), training energy expenditure, training volume (exercise minutes), serum hormones (PYY, ghrelin and TT₃), and daily urinary metabolites (estrogen, progesterone and LH) were collected during the study. The primary outcome measures pertaining to the current sub-study include detailed assessment of menstrual status by daily urinary hormones and measures of fasting total ghrelin concentrations (pg/ml), and fasting total PYY concentrations (pg/ml). Secondary outcome measures include body weight, fat mass, lean mass and TT₃.
<table>
<thead>
<tr>
<th>Screening</th>
<th>Baseline Week 3</th>
<th>Intervention Week 1</th>
<th>Intervention Week 5</th>
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**Figure 1.** The experimental design during screening, baseline, and six month intervention.
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. Inclusion criteria for this study were: 1) age 18-35 years, 2) body mass index (BMI) 16-25 kg/m², 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 binging globulin (SHBG) (nmol/L)) *100) [45] > 6, since an FAI greater than 6.0 has been reported to be consistent with hyperandrogenemia [46, 47]. In addition, an FAI greater than 6.0 represents values greater than three standard deviations from the mean of 1.6±0.3 in our reference population which consisted of healthy premenopausal exercising women (n=33) with documented ovulatory menstrual status by the assessment of daily urinary hormone measurement [1, 2].

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 [48, 49], bone health, and psychological health [50-53]. A physical exam was performed by an on-site clinician to determine overall health and check for physical signs of PCOS such as acne or hirsutism and symptoms of disordered eating. A fasting blood sample was analyzed for a complete blood count, basic chemistry panel, and an endocrine panel which included measures of LH, follicle stimulating hormone (FSH), 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 completed a semi-structured interview with each subject to exclude those women experiencing major psychiatric disorders including depression or clinical eating disorders. Participants met with a General Clinical Research Center (GCRC) 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. Additionally, dual-energy x-ray absorptiometry (DXA) scans of the total body, lumbar spine, and dual femur were performed to assess bone mineral density and body composition.

**Figure 2** depicts the progression of participants throughout the study. One hundred and sixty-four participants 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 12 for other reasons. Twenty-five women completed 6 months of the study. However, one subject with oligomenorrhea was not included in the recovery of menses (described below), therefore the analysis for recovery of menses is based on a sample size of twenty-four women.
Figure 2. A flow chart of the progression of participants through the study.
Baseline Procedures

During baseline participants daily urine and menstrual calendars were collected (see Classification of Baseline Menstrual Status). Additionally, participants underwent blood sampling for the determination of serum PYY, ghrelin, and TT3 (see Serum Hormone Analysis). Body weight was collected weekly. Participants completed a 3-day diet log and underwent, body composition (see Anthropometrics), and exercise testing (see Exercise Testing).

Classification of Baseline Menstrual Status

The initial classification of menstrual status prior to the intervention was based on self-reported menstrual histories, the results of a physical examination, urinary E1G, PdG, and LH profiles, and other endocrine measures described below. Participants were asked to record menstrual bleeding patterns and any additional symptoms related to menstrual cycles. Menstrual calendars were used to chart menstrual symptoms, such as cramps, bleeding, spotting, discharge, etc in all participants. Participants collected first morning urine samples throughout a 4 week baseline monitoring period and 6 month intervention period. Oligomenorrhea was confirmed if menses occurred at intervals of 36-90 days during the baseline period and if participants self reported 6 or less menstrual cycles in the past year prior to the intervention. FHA was assessed by confirming a negative pregnancy test, no menses in the past 90 days, and documentation of chronically suppressed E1G and PdG profiles observed during the baseline period.
**Intervention Procedures**

Participants randomly assigned to the treatment group (EAMD + calories) were asked to increase their caloric intake 20-30% above baseline energy requirements while maintaining their usual exercise training regime. Participants in the EAMD Control group were asked to maintain their baseline physical activity levels and food intake. 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. A registered dietician met with the participants 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 (PYY, ghrelin and TT3) and daily urinary metabolites (estrogen, progesterone, and LH) were collected during the intervention.

**Recovery of Menstrual Function Categories**
To describe the recovery of menstrual function, we grouped participants based on several definitions of recovery that ranged in physiological and clinical relevance. For each of the following 6 categories describing menstrual recovery, participants were deemed to either have met the recovery of menstrual function criteria during the intervention or have failed to meet the recovery of menstrual function criteria. The definitions used for each category differed somewhat between women deemed to exhibit functional hypothalamic amenorrhea vs. oligomenorrhea. The first category (Recovery Category 1) was described as “recovery of menses.” The successful recovery of menses in women with exercise associated FHA 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 self-reported frequency of menses 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 recovery of menses (Recovery Category 1) analysis because, although she had a 45 day baseline menstrual cycle and subsequently demonstrated six occurrences of menses during the intervention, she self reported 6 menstrual cycles in the last 6 months during her menstrual history.

For further analysis of the recovery of menstrual function, participants were assigned to Recovery Category 2 (recovery of menses preceded by increased estrogenic status) if the first occurrence of menses during the intervention was preceded by a
significant increase in estrogenic status defined as an increase in urinary estrone-1-glucuronide (E1G) concentrations above 35 ng/ml for at least 3 consecutive days. Seven women with oligomenorrhea were not included in this category tabulation because women with oligomenorrhea typically display elevated and erratic estrogen concentrations [1], and this was observed in our oligomenorrheic participants at Baseline. Recovery Category 3 (resumption of menses preceded by ovulation) was described as resumption of menses preceded by ovulation based on increases in urinary E1G (above 35 ng/ml), pregnanediol-3-glucoronide (PdG, above 2.5 μg/ml), and mid-cycle LH (above 25 mIU/ml) concentrations [1, 2]. Two women with oligomenorrhea who had ovulatory baseline cycles were not included in this category tabulation. Recovery Category 4 was described as resumption of menses followed by at least 2 menstrual cycles of less than 36 days each. Recovery Category 5 was described as resumption of menses followed by at least 2 menstrual cycles of less than 90 days each. The seven women with oligomenorrhea were also not included in this category as oligomenorrhea is defined as long and irregular menstrual cycles 36 to 90 days in length [1] and thus, this definition would not indicate “recovery”.

**Anthropometrics**

Total body weight was measured by a digital scale in the laboratory to the nearest 0.01 kg with participants wearing t-shirt and gym shorts during each week of the baseline period and intervention weeks 5, 9, and 21. Height was measured to the nearest 1.0 cm without shoes during the screening period and BMI was calculated as a ratio of weight to height (kg/m²) during each week of baseline and intervention weeks 5, 9, and 21. Baseline values for body weight and BMI were reported as the average of all baseline and
screening measurements. Body composition, including percent body fat, fat mass, fat
free mass, and lean body mass 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=7, GE Lunar Corporation,
Madison, WI, enCORE 2002 software, version 6.50.069) or a GE Lunar iDXA scanner
(n=15, 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, 14 participants were scanned in triplicate on both machines.
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. 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

Dietary energy intake was assessed from a 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 Testing**

Maximal aerobic capacity ($\text{VO}_2\text{max}$) was measured during a progressive treadmill test to volitional exhaustion 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 using indirect calorimetry. The VO$_2$ max test is initiated by the participant selecting a comfortable running speed at 0.0% grade. The grade of the treadmill was increased 2.0% after every 2 minutes for the first 8 minutes of the test, after which the grade then was increased 1.0% for each subsequent minute [11, 54, 55].

**Urinary Hormone Measurements**

All urine samples were corrected for specific gravity using a hand refractometer (NSG Precision Cells) to account for hydration status [56] which has been reported to perform as well as creatinine correction for adjusting urinary hormone concentrations [56]. The secretion of E1G and PdG metabolites in the urine parallels serum
concentrations of the parent hormones [57]. 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 [1, 58]. 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.

Mean 28 day E1G was calculated by subtracting recovery 28 day E1G from baseline 28 day E1G. Baseline mean 28 E1G was the mean of E1G during the 28 day baseline period. Recovery 28 day E1G was calculated as the E1G mean 28 days preceding the resumption of menses in the women with amenorrhea who resumed menses and was calculated as the six month 28 day E1G mean in all the oligomenorrheic women and in the amenorrheic women who did not resume menses.

**Blood Sampling**

Blood 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 GCRC nurse 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.

**Serum Hormone Analysis**

Total PYY was measured using a radioimmunoassay (RIA) for total PYY (Linco Research, St. Charles, MO). The total PYY assay recognizes both PYY$_{1-36}$ and PYY$_{3-36}$ and does not require the addition of inhibitors. The intra-assay and inter-assay coefficients of variation were 5.3 and 7.0%, respectively. The sensitivity of the assay was 10 pg/ml. Total ghrelin was measured by RIA for total ghrelin (Linco Research). The intra-assay and inter-assay coefficients of variation were 2.0 and 15.7%, respectively. The sensitivity of the assay was 93 pg/ml. TT$_3$ was analyzed using a chemiluminescence-based immunoassay analyzer (Diagnostic Products Corporation, Los Angeles, CA). Analytical sensitivity for the TT$_3$ assay was 35 ng/dl. The intra-assay and inter-assay coefficients of variation were 13.2% and 15.6%, respectively. All samples from a given subject were analyzed in duplicate.

**Statistical Analysis**

Data screening was conducted prior to analysis, involving outlier detection and examination of variable distributions within each of the groups for normality. An ANOVA with repeated measures and one grouping factor was performed to compare fasting total ghrelin concentrations, fasting PYY concentrations, and other variables of interest over time between the EAMD-R and the EAMD-NR groups. Baseline
measurements as well as change scores for other key variables were examined using independent t-tests. Pearson correlation coefficient analyses were used to examine the relationship between changes in total PYY, total ghrelin and other variables of interest. To explore the strongest predictors of change in mean E1G, we used stepwise linear regression, using p=0.05 for entry and p=0.010 to leave the model. To explore the strongest predictors of the recovery of menses categories 1, 2, 3, 4 and 5, we used forward stepwise logistic regression, using p=0.05 for entry and p=0.010 to leave the model. In all analyses, P<0.05 was considered statistically significant. Statistical analyses was performed using SPSS software (version 16.0; SPSS Inc., Chicago,IL). All data were reported as mean ± standard error.

A power calculation was performed to determine sample sizes required to detect differences. For fasting total ghrelin concentrations, sample size was based on the detection of a meaningful difference of 404 pg/ml and an SD of 499 pg/ml based on previously published reports in women with exercising women with amenorrhea compared to exercising women with ovulatory menstrual cycles [21]. In order to achieve 80% power for the total ghrelin analysis using a 0.05 level of significance, a sample size of 8 participants per group (total n= 16) will be required, for the analysis. For fasting total PYY concentrations, sample size was based on the detection of a meaningful difference of 52 pg/ml and an SD of 58 pg/ml based on previously published reports in women with exercising women with amenorrhea compared to exercising women with ovulatory menstrual cycles [21]. In order to achieve 80% power for the total PYY analysis using a 0.05 level of significance, a sample size of 7 participants per group (total n= 14) will be required, for the analysis.
RESULTS

Baseline Characteristics

Demographic characteristics of the exercising women with EAMD (n=24) at baseline are presented in Table 1. At baseline, based on Recovery Category 1 (the first occurrence of menstrual bleeding during the intervention), the EAMD-R and EAMD-NR groups were similar with respect to age (p=0.288), height (p=0.561), weight (p=0.320), and BMI (p=0.133). However, the EAMD-NR group had a lower percentage of body fat (p=0.018) and fat mass (kg) (p=0.021) compared to the EAMD-R group at baseline. There was no difference between the EAMD-R and EAMD-NR groups with respect to lean body mass (p=0.911), peak aerobic capacity (p=0.528), weekly physical activity minutes (p=0.846). The duration of amenorrhea (p=0.027) was longer in the EAMD-NR group (355.4±78.3 days) compared to the EAMD-R group (162.5±27.6 days, p=0.027).
Table 1. Demographic characteristics of the exercising women EAMD.

<table>
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<tr>
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<th>EAMD-NR (n=9)</th>
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<tr>
<td>Height (cm)</td>
<td>166.1±1.6</td>
<td>167.6±2.1</td>
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<td>Weight (kg)</td>
<td>58.5±1.7</td>
<td>55.5±2.5</td>
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<td>Body mass index (kg/m²)</td>
<td>21.2±0.4</td>
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<td>Body fat (%)</td>
<td>25.8±1.0</td>
<td>20.3±2.3</td>
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<td>Fat mass (kg)</td>
<td>15.1±0.7</td>
<td>11.3±1.5</td>
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<td>Lean body mass (kg)</td>
<td>41.1±1.2</td>
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<td>VO2peak (ml/kg/min)</td>
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<td>48.5±2.9</td>
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<td>Exercise volume (min/week)</td>
<td>397±72</td>
<td>374±91</td>
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<td>13.5±0.4</td>
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<td>Duration of Amenorrhea (days)</td>
<td>162.5±27.6</td>
<td>355.4±78.3</td>
<td><strong>0.027</strong></td>
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Values are the mean ± SEM.
EAMD-R - Women with severe exercise-associated menstrual disturbances who experienced menstrual recovery between months one and six of the intervention
EAMD-NR - Women with severe exercise-associated menstrual disturbances who did not resume menses within 6 months of the intervention
Independent T-tests were used to compare height, weight, body mass index, body fat, fat mass, lean body mass, and peak aerobic capacity.
Mann-Whitney U Tests were to compare age, exercise volume, and duration of amenorrhea.
Baseline hormonal concentrations of the exercising women with EAMD (n=24) at baseline are presented in Table 2. There was no difference between the EAMD-R and EAMD-NR groups with respect to baseline ghrelin (p=0.245), PYY (p=0.164), and TT₃ concentrations (p=0.899).

**Table 2.** Baseline hormonal characteristics of the exercising women with EAMD.

<table>
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<tr>
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<td>Ghrelin (pg/ml)</td>
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<td>PYY (pg/ml)</td>
<td>72.6±3.9</td>
<td>84.2±8.1</td>
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<td>TT₃ (ng/dl)</td>
<td>78.2±7.5</td>
<td>79.6±5.7</td>
<td>0.899</td>
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</table>

Values are the mean ± SEM.
TT₃ - total triiodothyronine
PYY – Peptide YY
EAMD-R - Women with severe exercise-associated menstrual disturbances who experienced menstrual recovery between months one and six of the intervention
EAMD-NR - Women with severe exercise-associated menstrual disturbances who did not resume menses within 6 months of the intervention
Independent T-tests were used to compare baseline PYY, and TT₃ concentrations.
Mann-Whitney U Tests were used to compare baseline ghrelin concentrations.

**Resumption of Menses**

The day and week of recovery of menses, based on categories 1-5 are presented in Table 3. Based on Recovery Category 1, the EAMD-R group experienced menstrual recovery on day 64.9±13.2 of the study intervention. Fifteen out of 24 women with EAMD experienced recovery of menses (Recovery Category 1). In 10 out of 17 women who experienced menstrual recovery, menses was preceded by a significant increase in estrogenic status (Recovery Category 2) 68.1±16.0 days into the intervention. In 9 out of
24 women who experienced menstrual recovery, menses was preceded by ovulation
(Recovery Category 3) on day 72.0±21.6 of the intervention. In 8 out of 25 women who
experienced menstrual recovery, menses was followed by at least 2 menstrual cycles of
less than 36 days in length (Recovery Category 4) on day 90.8±5.2 of the intervention. In
9 out of 17 women who experienced menstrual recovery, menses was followed by at least
2 menstrual cycles of less than 90 days in length (Recovery Category 5) on day
60.4±12.1 of the intervention.

Table 3. Resumption of menses, resumption of estrogenic status, resumption of
ovulatory function, resumption of regular menstrual cycle intervals (<36 days apart) and
resumption of regular menstrual cycle intervals (<90 days apart).

<table>
<thead>
<tr>
<th>Recovery Category</th>
<th>Days until resumption</th>
<th>Weeks until resumption</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>64.9±13.2</td>
<td>9.7±1.8</td>
</tr>
<tr>
<td>2</td>
<td>68.1±16.0</td>
<td>10.2±2.2</td>
</tr>
<tr>
<td>3</td>
<td>72.0±21.6</td>
<td>10.9±3.1</td>
</tr>
<tr>
<td>4</td>
<td>90.8±5.2</td>
<td>13.3±0.9</td>
</tr>
<tr>
<td>5</td>
<td>60.4±12.1</td>
<td>9.1±1.7</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM.

Recovery Category 1 was described as “recovery of menses.” The successful recovery of menses in women
with exercise associated amenorrhea at baseline was defined as the first occurrence of menstrual bleeding
during the intervention. 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 self-reported frequency of menses during the 6 months prior to the
intervention.

Recovery Category 2 or “recovery of menses preceded by increased estrogenic status” if the first
occurrence of menses during the intervention was preceded by a significant increase in estrogenic status
defined as an increase in urinary estrone -1-glucuronide (E1G) concentrations above 35 ng/ml for at least 3
consecutive days.

Recovery Category 3 or “resumption of menses preceded by ovulation” was described as resumption of
menses preceded by ovulation based on increases in urinary E1G (above 35 ng/ml), pregnanediol-3-
glucuronide (PdG, above 2.5 μg/ml), and LH (above 25 mIU/ml) concentrations

Recovery Category 4 was described as resumption of menses followed by at least 2 menstrual cycles of less
than 36 days.

Recovery Category 5 was described as resumption of menses followed by at least 2 menstrual cycles of less
than 90 days.
Example of profiles of E1G and PdG concentrations for baseline and 6 months of the intervention are demonstrated in Figure 3. Example subject #1 had a history of amenorrhea for 90 days and at day 72 of the intervention she experienced menstrual recovery that was preceded by estrogen activity (Recovery Category 1 and 2) and ovulation (Recovery Category 3). Example subject #1 also had 2 more regular menstrual cycles <90 days apart (Recovery Category 5). Example subject #2 had a history of amenorrhea for 720 days and was in the EAMD-NR group since she failed to resume menses during the intervention.
Figure 3. Example of profiles of urinary estrone-1-glucuronide (E1G) and pregnanediol-3-glucoronide (PdG) concentrations for 1 month of baseline and throughout 6 months of the intervention.
At baseline there was no difference between the EAMD-R and EAMD-NR (Recovery Category 1) groups (p=0.760) with respect to mean daily E1G concentrations. However, the mean E1G concentration 28 days preceding menses was elevated in the EAMD-R group compared to the EAMD-NR group (p=0.21) (Figure 4A). The percent change in mean E1G concentrations preceding menses (or at 6 months if menses was not resumed) was significantly different (p=0.001) in the EAMD-R group compared to the EAMD-NR group (Figure 4B). Figure 5 reflects the daily E1G concentrations during baseline and preceding menses (or at 6 months if menses was not resumed) of the women in the EAMD-NR group (Figure 5A and 5B) and in the EAMD-R group (Figure 5C and 5D).
Figure 4. (A) The absolute 28 day mean concentrations of urinary estrone -1-glucuronide (E1G) at baseline and preceding menses (or at 6 months if menses was not resumed). (B) The percentage change in mean E1G concentrations preceding menses (or at 6 months if menses was not resumed).
Figure 5
Energy Intake, Exercise, and Body Composition Changes During the intervention

During the intervention there was no significant (p=0.342) difference in the change in food intake measured by 3 day diet logs from baseline to 6 months. Exercise minutes increased (p=0.030) in the EAMD-R group compared to the EAMD-NR group.

Figure 6B demonstrates the change in body weight, fat mass and lean body mass during 6 months of the intervention in the EAMD-R group compared to the EAMD-NR group. The change in body weight (p=0.008) and lean body mass (p=0.011) from baseline to 6 months was significantly greater in the EAMD-R group compared to the EAMD-NR group, while there was no difference in the change in fat mass (p=0.313) during this same time period. When controlling for baseline group differences in fat mass, there was a significant difference in the change in body weight (p=0.001) and lean body mass (p=0.004) from baseline to 6 months in the EAMD-R group compared to the EAMD-NR group.

Figure 5. Daily urinary estrone -1-glucuronide (E1G) concentrations during baseline and preceding menses (or at 6 months if menses was not resumed) of the women in the EAMD-NR group (those who did not resume menses within 6 months of the intervention, Figure 5A and 5B) and in the EAMD-R group (those who experienced menstrual recovery between months one and six of the intervention, Figure 5C and 5D).
Figure 6 (A). The percentage change in ghrelin, peptide YY (PYY) and total triiodothyronine (TT₃) concentrations over 6 months. (B) The percentage change in body weight, fat mass and lean body mass over 6 months.
Hormonal Changes during the Intervention

Results from repeated measured ANOVAs over time exploring ghrelin concentrations during resumption of reproductive function based on Recovery Categories 1,2,3,4 and 5 are presented in Table 4. Baseline, Intervention week 5, 9, and 21 time points demonstrated a significant time by experimental group interaction for fasting total ghrelin concentrations (p<0.001), demonstrating that women in the EAMD-R group have decreases in total circulating ghrelin concentrations from baseline to 6 months, while the EAMD-NR group have increases in total ghrelin concentrations from baseline to 6 months. The percent change in ghrelin concentrations from baseline to 6 months was significantly different (p=0.001) in the EAMD-R group compared to the EAMD-NR group (Figure 6A). When controlling for baseline group differences in fat mass, there was a significant difference for the change in ghrelin (p=0.004) in the EAMD-R group compared to the EAMD-NR group. Figure 7A demonstrates the changes in ghrelin over 6 months expressed by percent change in the EAMD-R group compared to the EAMD-NR group (Recovery Category 1).
Table 4. Results from repeated measured ANOVAs over time exploring ghrelin concentrations during resumption of reproductive function based on resumption of menses, resumption of estrogenic status, resumption of ovulary function, resumption of regular menstrual cycle intervals (<36 days apart) and resumption of regular menstrual cycle intervals (<90 days apart)

<table>
<thead>
<tr>
<th>Recovery Category</th>
<th>Sample Size</th>
<th>Time x group interaction p-value</th>
<th>Time effect p-value</th>
<th>Group effect p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>YES group</td>
<td>No group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recovery Category 1</td>
<td>15</td>
<td>8</td>
<td>&lt;0.001</td>
<td>0.380</td>
</tr>
<tr>
<td>Recovery Category 2</td>
<td>10</td>
<td>7</td>
<td>0.001</td>
<td>0.152</td>
</tr>
<tr>
<td>Recovery Category 3</td>
<td>9</td>
<td>13</td>
<td>0.282</td>
<td>0.369</td>
</tr>
<tr>
<td>Recovery Category 4</td>
<td>8</td>
<td>16</td>
<td>0.101</td>
<td>0.264</td>
</tr>
<tr>
<td>Recovery Category 5</td>
<td>9</td>
<td>8</td>
<td>0.011</td>
<td>0.112</td>
</tr>
</tbody>
</table>

Recovery Category 1 was described as “recovery of menses.” The successful recovery of menses in women with exercise associated amenorrhea at baseline was defined as the first occurrence of menstrual bleeding during the intervention. 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 self-reported frequency of menses during the 6 months prior to the intervention.

Recovery Category 2 or “recovery of menses preceded by increased estrogenic status” if the first occurrence of menses during the intervention was preceded by a significant increase in estrogenic status defined as an increase in urinary estrone -1-glucuronide (E1G) concentrations above 35 ng/ml for at least 3 consecutive days.

Recovery Category 3 or “resumption of menses preceded by ovulation” was described as resumption of menses preceded by ovulation based on increases in urinary E1G (above 35 ng/ml), pregnanediol-3-glucuronide (PdG, above 2.5 μg/ml), and LH (above 25 mIU/ml) concentrations.

Recovery Category 4 was described as resumption of menses followed by at least 2 menstrual cycles of less than 36 days.

Recovery Category 5 was described as resumption of menses followed by at least 2 menstrual cycles of less than 90 days.
Figure 7 (A). The percentage change in ghrelin over 6 months in the EAMD-R (those who experienced menstrual recovery between months one and six of the intervention) group compared to the EAMD-NR (those who did not resume menses within 6 months of the intervention) group (Recovery Category 1). (B) The percentage change in peptide YY (PYY) over 6 months in the EAMD-R group compared to the EAMD-NR group (Recovery Category 1).
Results from repeated measured ANOVAs over time exploring PYY concentrations during resumption of reproductive function based on Recovery Categories 1,2,3,4 and 5 are presented in Table 5. Baseline, Intervention week 5, 9, and 21 time points did not demonstrate a significant time by experimental group interaction for fasting total PYY concentrations (p=0.173), time effect (p=0.857), or group effect (p=0.656) demonstrating that women in the EAMD-R group and EAMD-NR group had no significant changes in PYY from baseline to 6 months. The percent change in PYY concentrations was not significantly different (p=0.177) in the EAMD-R group compared to the EAMD-NR group from baseline to 6 months (Figure 6A). Figure 7B demonstrates the circulating PYY concentrations over 6 months expressed by percentage change in the EAMD-R group compared to the EAMD-NR group (Recovery Category 1).

Baseline, Intervention week 5, 9, and 21 time points did not demonstrate a significant time by experimental group interaction (p=0.077), time effect (p=0.215), or group effect (p=0.758) for fasting TT3 concentrations demonstrating that from baseline to 6 months of the intervention women in the EAMD-R group and EAMD-NR group had no significant changes in TT3 concentrations over time or when comparing groups. The percentage change in TT3 concentrations from baseline to 6 months of the intervention was not significantly different (p=0.132) in the EAMD-R group compared to the EAMD-NR group (Recovery Category 1, Figure 6A).
Table 5. Results from repeated measured ANOVAs over time exploring peptide YY concentrations during resumption of reproductive function based on resumption of menses, resumption of estrogenic status, resumption of ovulary function, resumption of regular menstrual cycle intervals (<36 days apart) and resumption of regular menstrual cycle intervals (<90 days apart).

<table>
<thead>
<tr>
<th>Recovery Category</th>
<th>Sample Size</th>
<th>Time x group interaction p-value</th>
<th>Time effect p-value</th>
<th>Group effect p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>YES group</td>
<td>No group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recovery Category 1</td>
<td>15</td>
<td>8</td>
<td>0.173</td>
<td>0.857</td>
</tr>
<tr>
<td>Recovery Category 2</td>
<td>10</td>
<td>7</td>
<td>0.065</td>
<td>0.897</td>
</tr>
<tr>
<td>Recovery Category 3</td>
<td>9</td>
<td>13</td>
<td>0.410</td>
<td>0.790</td>
</tr>
<tr>
<td>Recovery Category 4</td>
<td>8</td>
<td>16</td>
<td>0.692</td>
<td>0.734</td>
</tr>
<tr>
<td>Recovery Category 5</td>
<td>9</td>
<td>8</td>
<td>0.237</td>
<td>0.826</td>
</tr>
</tbody>
</table>

Recovery Category 1 was described as “recovery of menses.” The successful recovery of menses in women with exercise associated amenorrhea at baseline was defined as the first occurrence of menstrual bleeding during the intervention. 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 self-reported frequency of menses during the 6 months prior to the intervention.

Recovery Category 2 or “recovery of menses preceded by increased estrogenic status” if the first occurrence of menses during the intervention was preceded by a significant increase in estrogenic status defined as an increase in urinary estrone -1-glucuronide (E1G) concentrations above 35 ng/ml for at least 3 consecutive days.

Recovery Category 3 or “resumption of menses preceded by ovulation” was described as resumption of menses preceded by ovulation based on increases in urinary E1G (above 35 ng/ml), pregnanediol-3-glucuronide (PdG, above 2.5 μg/ml), and LH (above 25 mIU/ml) concentrations.

Recovery Category 4 was described as resumption of menses followed by at least 2 menstrual cycles of less than 36 days.

Recovery Category 5 was described as resumption of menses followed by at least 2 menstrual cycles of less than 90 days.
Associations with Gastrointestinal Hormones

There was a significant correlation between change in ghrelin and change in body weight \((r=-0.521, p=0.013)\), and a significant correlation between change in ghrelin and change in BMI \((r=-0.517, p=0.016)\) when all participants are combined from baseline to 6 months. There was not a significant correlation between change in ghrelin and change in PYY \((r=-0.316, p=0.152)\) from baseline to 6 months (Table 6). To explore the strongest predictors of change in ghrelin, we demonstrated using stepwise linear regression, that change in body weight \((r=0.353, p=0.019)\) was the strongest predictor accounting for 10.4% of the variability of the change in ghrelin.

Table 6. Bivariate correlations of change in ghrelin.

<table>
<thead>
<tr>
<th>Change in Ghrelin (n=24)</th>
<th>R value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in PYY</td>
<td>-0.316</td>
<td>0.152</td>
</tr>
<tr>
<td>Change in TT₃</td>
<td>-0.200</td>
<td>0.386</td>
</tr>
<tr>
<td>Change in body weight (kg)</td>
<td>-0.521</td>
<td>0.013*</td>
</tr>
<tr>
<td>Change in body mass index (kg/m²)</td>
<td>-0.517</td>
<td>0.016*</td>
</tr>
<tr>
<td>Change fat mass (kg)</td>
<td>-0.197</td>
<td>0.379</td>
</tr>
<tr>
<td>Change in fat free mass (kg)</td>
<td>-0.325</td>
<td>0.140</td>
</tr>
</tbody>
</table>

*Significant correlations \((p<0.05)\)
Note; all participants are included in the analysis.
**Associations with Estrogen**

There was a significant correlation between change in mean E1G from baseline to 6 months and in ghrelin from baseline to 6 months ($r=-0.539, p=0.010$), and a significant correlation between change in mean E1G from baseline to 6 months and BMI from baseline to 6 months ($r=0.624, p=0.002$) and body weight from baseline to 6 months ($r=0.616, p=0.002$) (**Figure 8, Table 7**). To explore the strongest predictors of change in mean E1G from baseline to 6 months, we demonstrated using stepwise linear regression, that change in body weight from baseline to 6 months ($r=0.599, p=0.005$) was the strongest predictor and predicts 32.3% of the variability of the change in mean E1G. To explore the strongest predictors of recovery of menses (Recovery Categories 1-5), using logistic regression variables that have previously been reported to be associated with reproductive function, such as changes in ghrelin, PYY, TT$_3$, body weight, BMI, fat mass, lean body mass from baseline to 6 months, and baseline fat mass were explored. The strongest independent predictor of recovery of menses was change in ghrelin from baseline to 6 months ($p=0.021$). No other categories of recovery of menses (Recovery Categories 2-5) had significant predictors.
Table 7. Bivariate correlations of change in mean 28 day E1G.

<table>
<thead>
<tr>
<th></th>
<th>R value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in Ghrelin</td>
<td>-0.539</td>
<td>0.010**</td>
</tr>
<tr>
<td>Change in PYY</td>
<td>0.198</td>
<td>0.376</td>
</tr>
<tr>
<td>Change in TT&lt;sub&gt;3&lt;/sub&gt;</td>
<td>0.202</td>
<td>0.368</td>
</tr>
<tr>
<td>Change in body weight (kg)</td>
<td>0.616</td>
<td>0.002**</td>
</tr>
<tr>
<td>Change in body mass index (kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>0.624</td>
<td>0.002**</td>
</tr>
<tr>
<td>Change fat mass (kg)</td>
<td>0.439</td>
<td>0.036*</td>
</tr>
<tr>
<td>Change in fat free mass (kg)</td>
<td>0.443</td>
<td>0.034*</td>
</tr>
</tbody>
</table>

*Significant correlations (p<0.05)

**Significant correlations (p<0.01)

Figure 8. Scatterplot of all subjects demonstrating the association between change in 28 day mean urinary estrone -1-glucuronide (E1G) and change in body weight.
DISCUSSION

This study is the first intervention to utilize dietary strategies targeting improvements in energy status as a non-pharmacological strategy to reverse amenorrhea in exercising women. During the 6 month intervention several novel findings were demonstrated. Firstly, we demonstrated that women with EAMD who experienced menstrual recovery experienced significant decreases (12% decrease) in fasting ghrelin concentrations compared to women with EAMD who did not resume menses (26% increase) (Recovery Category 1). These are important findings since we speculate that elevated ghrelin concentrations may suppress reproductive function in women with menstrual cycle disturbances and may be mechanistically important during the resumption of menses. We propose that the demonstrated decreases in circulating ghrelin concentrations observed during our refeeding protocol were mediated by the favorable 3% change in body weight demonstrating the importance of energetic milieu to reproductive recovery.

More specifically, ghrelin concentrations were decreased in the EAMD women who successfully experienced menstrual recovery when menses was preceded by improved estrogenic exposure (Recovery Category 2) and when initial menses was followed by at least 2 additional menstrual cycles (Recovery Category 5). These findings are important because they may indicate that decreases in circulating ghrelin concentrations are associated with more advanced reproductive recovery more so than simply the onset of menses. These findings presumably demonstrate that recovery of menses in these situations is likely associated with successful follicular development secondary to recovery of metabolic factors, such as increased ghrelin and TT3 (and other
improvements not reported herein such as increased leptin and IGF-1) that are associated with elevations in circulating estrogen concentrations secondary to successful follicular development. In this data set, urinary estrogen exposure was increased by 55% in the women who resumed menses. However, we did not demonstrate that change in ghrelin concentration was related to resumption of menses preceded by ovulation (Recovery Category 3), indicating that either 1) more time is needed for further recovery of metabolic factors associated with advancing follicular development to initiate ovulation, or 2) other metabolic and growth factors not measured in the current study, must be “normalized” before resumption of ovulatory function can occur.

Despite fasting ghrelin concentrations consistently being demonstrated to be elevated in exercising women with FHA [11, 14, 19, 21, 22, 59], this is the first study to demonstrate that decreases in ghrelin concentrations related to favorable changes in body weight, are associated with recovery of menses in exercising women with EAMD. Changes in ghrelin are tightly linked to changes in body weight and energy availability [60, 61] and in the current study the decreases in circulating ghrelin concentrations were associated with increases in body weight of 3 percent. We also speculate that the changes in ghrelin were likely dependent on increases in energy availability and body weight and, while ghrelin changes may be mechanistically important for recovery of menses, increases in body weight are a key clinical outcome to monitor during the recovery of menses. Weight gain has also been demonstrated to decrease both fasting and postprandial ghrelin concentrations in women with anorexia nervosa [34], supporting the notion that changes in ghrelin are tightly related to changes in body weight.
In this 6 month intervention designed to increase caloric intake in exercising women with EAMD, an important strength of our study design is the in depth strategies used to categorize menstrual function. We categorized resumption of menses based on several definitions of recovery of reproductive function that ranged in physiological and clinical relevance that not only referenced resumption of menses, but also referenced estrogenic status, ovulation, and inter-menstrual intervals. It is interesting that the time course necessary to resumption of menses and ovulation was longer than the time course to the first onset on menses, and even longer for three cycles of typically normal cycle length to occur. These findings may indicate that the first menses following amenorrhea is not always preceded by ovulation. In our exercising women with FHA only 5 out of 11 women who experienced menstrual recovery had menses that was preceded by ovulation, indicating that more time and/or improvements in energetic status may be necessary to resume ovulatory function. Energetic and nutritional factors have profound effects on follicular growth and development and can be correlated with changes in circulating concentrations of metabolic hormones, including insulin, IGF-I, GH, and leptin [28].

In chronically energy deficient women, i.e. women with anorexia nervosa, improving energy status and increasing body weight has been demonstrated to be a key factor associated with resumption of menses [31-33]. Golden et al. [31] demonstrated that adolescents with anorexia nervosa required a weight gain of ~2 kg or greater than the body weight at which menses was lost in order for the resumption of menses to occur. To this end, in adolescents with eating disorders, there is a strong association between weight at return of menses and the weight at last menses [33], indicating that while treatment goals need to be individualized [32], weight gain is an essential means to
improve energy status. Of additional clinical importance, Miller and colleagues [62] demonstrated that at least 10% weight gain in women with anorexia nervosa resulted in increased hip bone mineral density and that recovery of menses, independent of increases in weight, resulted in increased spinal bone mineral density. The results from Miller and colleagues [62] emphasize the importance of recovery of menses and increasing estrogen exposure to improved bone mineral density.

In our laboratory, (Scheid, submitted to AJP, 2012) we prospectively demonstrated a relationship between elevated ghrelin concentrations and decreased LH pulse frequency in premenopausal women following diet- and exercise-associated weight loss, indicating a relationship between circulating ghrelin concentrations and reproductive regulation. The current study supports the association between ghrelin and reproductive function in premenopausal women since we demonstrate a relationship between decreases in ghrelin and resumption of menses. However, assessing menstruation in premenopausal women is a more clinically relevant measure of reproductive function compared to LH pulse frequency and, in the current study, for the first time, we demonstrate that decreases in ghrelin may be necessary, likely in conjunction with changes in other metabolic hormones, for exercising women with FHA to resume menses.

Decreased circulating ghrelin concentrations in women with successful resumption of menses may be mechanistically involved in the resumption of menses via hypothalamic signaling. In animal models, many investigators have demonstrated a direct relationship between elevated ghrelin concentrations and reproductive suppression [37-39]. Vulliemoz et al. [38] infused ghrelin in rhesus monkeys and demonstrated that
ghrelin infusion decreased LH pulse frequency. In men, investigators [35, 36] have demonstrated that LH secretion is suppressed following ghrelin administration. While in the current study we cannot determine if decreases in ghrelin directly impact LH pulsatility, we speculate that ghrelin is likely an important signal involved in the resumption of menses in women with EAMD.

While the current study does not support the notion that changes in PYY concentrations are involved in the resumption of menses in EAMD women, we have consistently demonstrated that exercising women with FHA have elevated fasting concentrations of PYY [21, 22]. Elevated fasting PYY concentrations are proposed to be related to an energy deficiency, as demonstrated by the negative correlations of PYY with BMI [63] and REE [21]. However, the results from the current study suggest that PYY is not altered after 6 months of a refeeding intervention in women with EAMD who resume menses, and gain weight, presumably indicating that PYY concentrations are not related to the recovery of menses. Since the change in PYY concentrations during this 6 month study was not correlated to the change in body weight, we speculate that PYY appears does not play a role in the modulation of body weight or reproductive function in women recovering from severe menstrual cycle disturbances.

Interestingly, Misra et al [64] suggested that elevated PYY may be involved in the pathogenesis of anorexia nervosa and presumably the energy deficiency observed in exercising women with FHA [21]. To this end, it has been suggested that PYY may be genetically predetermined as opposed to related to alterations in energy availability or weight loss [65]. Using systematic polymorphism and discovery and genotyping, Shih et al. [65] demonstrated that there is genetic variation of the PYY locus, that PYY is highly
heritable, and that PYY shares a significant genetic determination with BMI. As such, we propose that having predetermined circulating PYY concentrations may cause the following problems in women with EAMD: 1) elevated PYY may cause decreases in hunger in women with EAMD since PYY is a known circulating satiety factor [66], compounding the eating behavioral phenotype consistent with the conscious restraint of food intake, a preoccupation with body weight and body shape, fear of gaining weight, and an association of achieved thinness with self esteem and self worth demonstrated in women with EAMD [67, 68], and 2) elevated PYY concentrations may cause an elevated thermic effect of food, since elevated PYY has been associated with elevated metabolism after a meal [69], leading to increased energy expenditure and contributing to the energy deficiency that women with EAMD must overcome in order to resume menses. While PYY concentrations in the current study were not significantly altered in the women who experienced menstrual recovery compared to the women who did not resume menses, PYY concentrations may be a contributing factor to an energy deficiency in women with EAMD, but may not be directly involved in reproductive suppression.

This current study design has notable strengths; the first and most notable strength is that we categorized participants based on several definitions of recovery of menses that ranged in physiological and clinical relevance. To accomplish this task, we had to carefully characterize daily reproductive hormone profiles of women with EAMD, including daily analysis of EIG, PdG, and LH followed by categorization of resumption of menses using five different definitions. Additionally multiple fasting blood draws occurred during the study to allow for repeated measures analysis of circulating ghrelin, PYY, and TT3.
While there were important strengths to the current study, there are some limitations to these data. The first limitation of the current study is that only fasting concentrations of ghrelin and PYY were measured. However, in our laboratory, we have confirmed strong correlations between fasting PYY and the post prandial peak in PYY, suggesting that fasting PYY relates directly to PYY concentrations after a meal [70]. To this end fasting ghrelin concentrations have also been demonstrated to be correlated with the ghrelin nadir following a meal [71]. Both of these studies indicate that the post-meal PYY and ghrelin concentrations likely observed in our population of women would be relative to the observed fasting PYY and ghrelin concentrations. Additionally, we also want to acknowledge that the hormones included in this study are not the only metabolic hormones that have been identified in the literature to directly, or indirectly affect reproductive function, including, but not limited to, leptin [72-75], insulin [74, 75], IGF-1 [76] and corticotropin-releasing hormone [77]. Specifically, leptin concentrations are also likely important for the resumption of menses in exercising women with FHA [72, 73, 78, 79]. However, only ghrelin, PYY, and TT₃ were measured in the current study. Additionally in the current study, there were two potentially impactful baseline differences between the women who experienced menstrual recovery within 6 months and the women who did not experienced menstrual recovery within 6 months: baseline fat mass and duration of amenorrhea before the study. The baseline differences in the two groups were minimized by analysis of covariance; however, the physiological impact of differences in baseline body composition and duration of amenorrhea may still have impacted the results of the current study. Finally, since the current study was a secondary analysis, we re-categorized women into one of two groups regardless of their initial group
assignment and there were 6 women in the EAMD-R group originally in the EAMD control group who were instructed to maintain their baseline energy intake. Therefore we speculate that these women may have resumed menses for one of the following reasons (1) increased caloric intake independent of the intervention, (2) decreased exercise energy expenditure (possibly caused by injury or change in seasonal training), or (3) spontaneous ovulation not caused by changes in energy status. Additionally there were 3 women in the EAMD-NR group originally in the EAMD+Calories group who were instructed to increase their energy intake and we speculate that these women were either non-compliant to the intervention or had another non-energy related suppression of the hypothalamic pituitary ovarian axis, such as stress.

In the current 6 month longitudinal study we demonstrated that circulating ghrelin concentrations were decreased in women who experienced menstrual recovery compared to the women who did not resume menses, indicating that elevated ghrelin may be mechanistically involved in regulating reproduction in women with EAMD. The changes in ghrelin that we observed in the current study are likely mediated by changes in body weight. Additionally, we demonstrated that ghrelin concentrations were decreased in EAMD women who successfully resumed menses preceded by an increased estrogenic exposure and in women who resumed menses followed by at least 2 menstrual cycles less than 90 days during 6 months, indicating that decreases in circulating ghrelin concentrations, related to increases in energy status and body weight, are associated with initial increases in reproductive function, including menstrual bleeding and elevations in circulating estrogen concentrations. However, we did not demonstrate that the change in ghrelin concentrations were related to resumption of menses preceded ovulatory function.
Additionally, PYY was not altered after 6 months in women with EAMD who resume menses, and gain weight, presumably indicating that PYY concentrations are not related to the recovery of menses. Future studies will need to attempt to understand if longer times periods are needed to see changes in ovulatory function or if other metabolic hormones are involved in the resumption of ovulatory function.
REFERENCES


CHAPTER VII

CONCLUSIONS

Ghrelin and Peptide YY (PYY) are both gastrointestinal peptides; PYY is secreted from the endocrine L cells of the ileum of the intestine [1, 2], while ghrelin is secreted by distinct endocrine cells of the stomach called X/A-like cells or ghrelin cells [3]. Both ghrelin and PYY appear are involved with appetite-regulation and energy homeostasis. Specifically, ghrelin concentrations respond rapidly to a negative energy balance, induced by either small meals or exercise [4]. Fasting ghrelin concentrations are elevated following weight loss due to a low calorie diet [5, 6] and combined diet and exercise [7, 8], demonstrating a role for ghrelin in the regulation of long-term energy homeostasis. Although PYY appears to play an important role in short-term energy homeostasis [9], signaling satiety [10] and potential meal termination [11], the evidence supporting PYY in a long-term role in energy balance regulation is conflicting. The primary purpose of this dissertation was to understand the physiological relevance of alterations in ghrelin and PYY in healthy populations of premenopausal women. The specific goals of this dissertation were to: (1) understand if PYY and ghrelin are involved in the long-term regulation of body weight in normal weight premenopausal healthy women (Study 1), (2) to understand if alterations in PYY and ghrelin are related to reproductive function in premenopausal healthy women (Study 2 and 4), and (3) to determine if college-aged women with different eating behavioral phenotypes, i.e., high vs normal dietary restraint, differ with respect to circulating concentrations of gastrointestinal hormones during and following a test meal (Study 3).
Study 1 was designed to examine changes in fasting total PYY and ghrelin in non-obese premenopausal women following an exercise and diet program with and without weight loss. We demonstrated that neither fasting ghrelin nor PYY changed in response to exercise training in the absence of weight loss, and PYY did not change with exercise and weight loss. Secondly we demonstrated that exercising women who lost weight increased circulating ghrelin concentrations. Additionally, the change in ghrelin during the intervention was inversely correlated with the change in body weight, body mass index, fat-free mass and energy availability, while the change in PYY was not correlated with any parameters of body weight or body composition. Therefore we were able to concluded that circulating ghrelin is sensitive to changes in body weight and energy availability *per se*, but fasting total PYY appears is not involved in the modulation of body weight or energy balance in premenopausal healthy women; Circulating PYY concentrations is an unlikely candidate for the regulation of long-term energy balance in healthy subjects.

While a clear role for ghrelin regulating energy homeostasis has been established, evidence has been accumulating indicating that ghrelin may also impact reproductive function through central actions on hypothalamic neurons in the arcuate nucleus causing indirect alterations on gonadotropin-releasing hormone (GnRH) neurons [12, 13]. We previously established (Study 1) that ghrelin concentrations are elevated in women who lost weight during an exercise and diet intervention compared to women who did not exercise and maintained body weight. Study 2 was designed to examine if elevations in 24 hour circulating ghrelin concentrations following a ~3 month exercise and diet program associated with diet- and exercise-induced weight loss are associated with a
decrease in luteinizing hormone (LH) pulsatility in premenopausal women. In the second study, we did indeed demonstrate that elevated total ghrelin concentrations were associated with the suppression of LH pulsatility in premenopausal women. We speculate that ghrelin is likely able to impact LH pulse frequency by altering concentrations of neuropeptide Y and agouti-related protein and the pro-opiomelanocortin and cocaine- and amphetamine regulated transcript in the arcuate nucleus, either by crossing the blood brain barrier or by signals transmitted by the vagus nerve to affect appetite and, indirectly by impacting the hypothalamic-pituitary-ovarian axis [12, 14]. Our findings suggest that the changes in ghrelin associated with an energy deficiency are also associated with the change in LH pulse frequency in premenopausal women and may play a role in the suppression of reproductive function.

The purpose of Study 3 was to determine if college-aged women with different eating behavioral phenotypes, i.e., high vs normal dietary restraint, differ with respect to circulating concentrations of gastrointestinal hormones during and following a test meal. We assessed the biologically active components of ghrelin and PYY, active ghrelin and PYY\textsubscript{3-36}, and these hormones were assessed before and after a meal at multiple time points. Interestingly, we demonstrated that PYY\textsubscript{3-36} concentrations were suppressed in the women with high dietary restraint compared to the women with normal dietary restraint. Low PYY\textsubscript{3-36} concentrations following a test meal may be indicative of a blunted appetite reduction after a meal and, over time, lead to an increased risk of weight gain; therefore, the results of Study 3 suggest that women with the eating behavior phenotype of elevated dietary restraint may be compensating for reduced physiological
signaling of PYY₃₋₃₆ circulating concentrations after a meal by consciously restraining food intake.

The purpose of Study 4 was to determine if ghrelin and PYY are associated with the recovery of menses in women with exercise associated menstrual cycle disturbances (EAMD) during an intervention of increased caloric intake. Recall in Study 2, we prospectively demonstrated a relationship between elevated ghrelin concentrations and decreased LH pulse frequency in premenopausal women following diet- and exercise-associated weight loss, indicating a relationship between circulating ghrelin concentrations and reproductive regulation. However, in Study 4 we assessed reproductive function by measuring recovery of menstrual function in women with EAMD instead of measuring LH pulse frequency. Assessing recovery of menstrual function in premenopausal women is a more clinically relevant measure of reproductive function compared to LH pulse frequency. In Study 4, we demonstrated that women with EAMD who experienced menstrual recovery experienced significant decreases in fasting ghrelin concentrations compared to women with EAMD who did not resume menses. We speculate that decreases in ghrelin may be necessary, likely in conjunction with changes in other metabolic hormones, for exercising women with EAMD to recover menstrual function. Increased caloric intake in exercising women with amenorrhea has been demonstrated to reverse amenorrhea [15, 16]. However, this was the first study to date, in humans to demonstrate reversal of low energy availability on ghrelin concentrations during the resumption of menses in exercising women with EAMD. Both Study 2 and Study 4 demonstrate a relationship between changes in ghrelin and changes in reproductive function. In both studies (Study 2 and Study 4) we could not determine
if changes in ghrelin directly impact LH pulsatility or recovery of menses, however, we speculate that ghrelin is likely an important signal involved the regulation of the hypothalamic pituitary ovarian axis in premenopausal women. Overall, ghrelin is likely an important signal involved the regulation of the hypothalamic pituitary ovarian axis in premenopausal women, while PYY after a meal is related to eating behavior phenotypes.

**FUTURE DIRECTIONS**

In Study 1, we attempted to understand if PYY and ghrelin are involved in the long-term regulation of body weight in normal weight premenopausal healthy women. While we demonstrated that exercising women who lost weight increased circulating ghrelin concentrations, we did not explore the compensatory changes that may have occurred following the elevated ghrelin concentrations, i.e. elevated food intake or decreased energy expenditure that would have occurred to compensate for the elevations in ghrelin following the energy deficit. An appropriate follow-up study to the first study would be a well-controlled weight loss study with a follow-up period exploring the consequences of elevated ghrelin (food intake and/or changes in body weight).

In Study 2, for the first time in women, we demonstrated that changes in ghrelin associated with an energy deficiency are also associated with the change in LH pulse frequency. However, we could not assess the direct impact of ghrelin of LH pulse frequency. Another study that explored the direct effect of elevated ghrelin on reproductive function in women [17] failed to demonstrate any effect on LH pulsatility. However, other investigators [18] have demonstrated that pulsatile LH secretion is suppressed in men following prolonged ghrelin administration, presumably indicating that chronic elevation in ghrelin is necessary to elicit a suppression of LH pulses. Kluge
and colleagues [18] demonstrated a decrease in LH pulse frequency and amplitude following four ghrelin injections (50µg) over a 12 hour period. Similarly, Lanfranco and colleagues [19] demonstrated that an 8-hour administration of acylated ghrelin decreased LH pulsatility. Both of these studies indicate that elevated levels of circulating ghrelin concentrations can inhibit reproductive function by decreasing LH pulse frequency in men, but these important follow-up studies have yet to be conducted in women. To determine if elevated ghrelin concentrations, independent of changes in body weight or energy availability, cause a suppression of LH pulse frequency in women, a future direction would be to infuse ghrelin over at least a 12 hour period to demonstrate if the elevations in circulating ghrelin caused a decrease in LH pulse frequency.

The results of Study 3 suggested that elevated cognitive restraint in college-aged women is a compensatory mechanism as a result of suppressed PYY3-36 circulating concentrations after a meal. Future studies need to investigate the relationship between eating behavioral phenotypes, PYY, and change in body weight over time. More specifically, it would be interesting to investigate PYY and cognitive restraint longitudinally to see if either of these food intake factors changes over time, and to demonstrate if these changes are associated with changes in body weight and to explore if changes in BMI over time are more strongly predicted by eating phenotypes or by gastrointestinal peptides like PYY. Additionally, in Study 3, we studied a very homogenous population of young college aged women. Future studies need to investigate PYY and cognitive restraint in many different populations of women, i.e. normal weight women, obese women, and women with anorexia nervosa, to investigate if PYY the interactions of PYY and cognitive restraint in different populations.
In Study 3, BMI and PYY$_{3-36}$ 180 min after the test meal predict 20.2% of the variability in dietary restraint. PYY concentrations around 3 hours following a meal may have important implications regarding the next meal or snack. An interesting future direction would be to measure behavioral outcomes such as ad libitum food intake 3 hours following the test meal to measure if the concentrations of PYY or cognitive restraint are more powerful predictors of food intake.

In Study 4, we demonstrated that ghrelin concentrations were decreased in women who resumed menses, estrogenic status and ovulatory function. The next important study to publish will be the intent to treat analysis, of ghrelin and PYY, from the clinical trial from which this sub-study was derived. The large clinical trial was designed to determine whether a 12-month intervention of increased caloric intake would improve indices of bone health and menstrual status in premenopausal women who suffer from severe EAMD. Women with EAMD were randomly assigned to either an increased caloric intake treatment group (EAMD+Calories) or a control group (EAMD Control). During the 12-month intervention, volunteers in the EAMD+Calories group followed modified dietary plan designed to achieve and maintain a target level of 20-30% above their previously determined baseline energy intake, in an effort to achieve a chronic energy surplus of +20-30% over their baseline energy requirements. Volunteers in the EAMD Control group were asked not to modify activity levels or food intake. The results from this important clinical trial will allow us to see if decreases in ghrelin can occur by increasing caloric intake and if the decreases in ghrelin precede the resumption of menses.
Another future direction of Study 4 is to determine if circulating leptin concentrations are altered by improving energy status via increased caloric intake. While it is likely that many factors modulate reproduction and signal adequate energy status to the arcuate nucleus, a critical leptin environment is likely a key signal for reproductive function in humans [20-22]. One important question in the clinical trial discussed above will be investigating what the strongest predictors of the resumption of menses are in EAMD women who resume menses. Will the change in leptin be important? Will the change in body fat be important? Will the change in ghrelin be important? Or will a number of factors contribute equally to the resumption of menses?

Once we confirm that increasing caloric intake causes important energetic and metabolic alterations leading to the recovery of reproductive function, follow up studies will need to establish the most effective dietary strategy to increase caloric intake, i.e. how many calories should a women increase caloric intake by? What percentage of calories should a women increase caloric intake by? Are some macronutrients more effective in increasing calories leading to recovery of menstrual function?

One strategy that may be an effective way to increase caloric intake in women with EAMD may be counseling them to increase the energy density in their diet. Reed et al. [23] demonstrated that women with EAMD consume a diet lower in energy density than regularly menstruating women. Additionally, Reed et al [23] found that vegetable and condiment intake (particularly low fat condiment intake) is increased in EAMD women, indicating that higher fat condiment intake may be one strategy to increase caloric intake in EAMD women. Overall the consumption of low energy dense foods may be a strategy to increase caloric intake in EAMD women.
REFERENCES


Curriculum Vitae

EDUCATION

2012  Ph.D. in Exercise Physiology, Pennsylvania State University
2007  M.Sc. in Exercise Science, University of Toronto
2005  B.Sc. in Kinesiology, University of Western Ontario

SELECTED AWARDS AND HONORS

2011  2011 Endocrine Trainee Day at the Endocrine Society
2009  CIHR Doctoral Research Award
2009  Endocrine Society Presidential Poster Competition
2008  The Endocrine Society Travel Grant
2007  ASBMR Student Travel Award
2007  CIHR: Canada Graduate Scholarships Doctoral Award
2005  James Edwards Melbourne Flin Award in Kinesiology (Athletic Leadership)

SELECTED PUBLICATIONS (6 OF 10)


