EFFECT OF ESTROGEN ON TEMPORALLY TARGETED FOOD RESTRICTION: ROLE OF GASTRIC, HORMONAL AND METABOLIC FACTORS

By

REBECCA J. NAUKAM

Bachelor of Science in Physiological Sciences
Oklahoma State University
Stillwater, Oklahoma
1976

Master of Theological Studies
Phillips Theological Seminary
Tulsa, Oklahoma
2010

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
May, 2015

EFFECT OF ESTROGEN ON TEMPORALLY TARGETED FOOD RESTRICTION: ROLE OF GASTRIC, HORMONAL AND METABOLIC FACTORS

Dissertation Approved:

Kathleen Curtis, PhD

Dissertation Adviser

Bruce Benjamin, PhD

Kenneth Miller, PhD

Shelia Kennison, PhD

Name: REBECCA NAUKAM

Date of Degree: MAY 2015

Title of Study: EFFECT OF ESTROGEN ON TEMPORALLY TARGETED FOOD RESTRICTION: ROLE OF GASTRI, HORMONAL AND METABOLIC FACTORS

Major Field: BIOMEDICAL SCIENCES

Abstract:

These studies used targeted temporal food restriction (TTFR; access to chow limited to two hours/day) in rats to model the eating pattern of some obese people who restrict their consumption of food to the evening, when activity is typically less, and eat little or no breakfast or lunch. I tested the hypothesis that Estradiol benzoate (EB)-treatment suppresses feeding and decreases body weight during TTFR, as it does during *ad libitum* feeding conditions, and investigated central nervous system (CNS) and peripheral mechanisms of these effects in TTFR rats that were fed or fasted on test days.

Ovariectomized (OVX) rats lost weight during TTFR, but the weight loss was greater in EBtreated rats. However, both EB-treated and oil vehicle-treated (OIL) control rats ate comparable amounts. To investigate central mechanisms of EB effects during TTFR, I examined neural interactions in the hindbrain nucleus of the solitary tract (NTS), where vagal afferents from the gastrointestinal tract terminate. cFos labeling in the NTS was increased in TTFR-fed rats, indicating neuronal activation associated with consumption of a meal. However, cFos immunolabeling was not affected by EB treatment. Moreover, numbers of the α subtype of estrogen receptors in the NTS were not affected by hormone treatment or feeding condition.

To investigate peripheral mechanisms underlying EB effects during TTFR, we assessed carbohydrate and fat metabolism, and related hormones. Plasma insulin was not different between hormone groups. Plasma glucose was in the physiological range, though lower in EB-treated rats. Liver glycogen was at physiological levels in *ad libitum* conditions, decreased as expected in TTFR-fasted groups, and was partially restored in TTFR-fed groups. Plasma leptin was not different between hormone groups, decreased as expected in TTFR-fasted groups, and then was partially restored in TTFR-fed groups. Plasma triglycerides (TG) and plasma free fatty acids (FFA) were elevated in EB-treated rats. Adrenal weights and plasma corticosterone were greater in EBtreated rats, which may indicate stress associated with TTFR. In addition, fat metabolism is affected by corticosterone, which may explain EB effects on TGs and FFAs and, thus, the differences in body weight during TTFR despite the lack of effect on food intake.

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CHAPTER I

INTRODUCTION

Research into the controls of food intake and body weight is increasingly important as the number of overweight and obese Americans continues to rise. Body weight increases substantially in women after menopause (100), an observation that suggests a role for ovarian hormones in body weight regulation. Unfortunately, the strategy many women employ to lose weight—decreasing the amount of calories and/or the number of meals consumed—has limited success, particularly over the long-term. Better understanding of the mechanisms by which ovarian hormones affect body weight during periods of food restriction, therefore, has the potential to provide critical insights into maintaining healthy body weight. Studies using animal models have facilitated understanding of the role of ovarian hormones in body weight regulation, and many focus on the interactions between body weight and food intake. In reproductively cycling female rats, body weight and food intake fluctuate with fluctuations in ovarian hormones (48, 52), with decreased body weight and food intake at proestrus when estradiol is greatest. Similarly, exogenous estradiol administration to *ad libitum* fed ovariectomized (OVX) rats decreases both food intake and body weight (64, 168).

Although the control of feeding is complex and multifaceted, estradiol appears to reduce feeding by altering peripheral signals, such as leptin, glucose and gastric distention, that inhibit food intake or by altering the sensitivity to such signals by the central nervous system (CNS).

Vagal afferents from the gastrointestinal (GI) tract terminate in the nucleus of the solitary tract (NTS) (33). Understandably, then, many investigations that sought to determine whether estradiol increases inhibitory signals and/or alters the sensitivity to such signals have focused on the NTS. Feeding-induced neural activation in the NTS is enhanced by estradiol benzoate (EB) treatment (50), suggesting that estradiol does, in fact, augment inhibitory signaling from the gut. Moreover, EB effects on feeding and body weight appear to involve binding to the α subtype of the estrogen receptor (ERα) at CNS sites involved in feeding, such as the NTS (115, 162). ERα are also located in the hypothalamus and other brain sites implicated in food intake and body weight. There is a lack of consensus about which of these sites is critical for the EB effects on feeding and body weight, though the NTS is a likely candidate in these effects.

In general, previous investigations of estradiol effects on feeding were conducted in rats that had *ad libitum* access to chow. These studies have provided a wealth of information about estradiol modulation of the controls of feeding in conditions when chow is freely available (10, 22, 49, 64). However, limiting food intake to reduce caloric intake is common in diets and other weight loss programs (57, 108, 146). Indeed, many obese dieters voluntarily restrict food intake and consume only a single large evening meal each day (14, 46, 159).

Several studies focused on different aspects of food intake and body weight and showed that male rats adapted to temporally targeted food restriction (TTFR) via changes in gastric empting or a shift to lipid metabolism with prolonged TTFR. With prolonged TTFR, metabolic changes occur that increase lipogenesis in liver and fatty tissue (94) and decrease hepatic glycogen depletion (93). Female rats have been subjected to TTFR in various feeding protocols (feeding for 2-4hr one or more time per day) (123, 128, 160, 163). These studies focused on the advantage of TTFR-induced diminished insulin release in the progression of breast tumors, the effects of mealfeeding on lipogenesis in brown adipose tissue and glycogen and glucose synchronization to

the phases of the circadian cycle in TTFR. However, estrogen effects on the metabolic factors during TTFR affecting body weight and food intake have not been investigated in female rats.

The prevalence of the TTFR strategy for obese and overweight women raises the possibility that estradiol may also impact feeding and body weight when food intake is restricted to a single time period each day (i.e. TTFR). Accordingly, we hypothesized that estradiol inhibits feeding and reduces body weight in rats during TTFR, as it does during *ad libitum* conditions. Therefore our goals were to investigate central and peripheral mechanisms of EB effects during TTFR. To assess central detection of signals related to food intake and body weight, we examined neural interactions in the hindbrain nucleus of the solitary tract (NTS), where vagal afferents from the gastrointestinal tract terminate. We also investigated peripheral mechanisms underlying EB effects on food intake and body weight during TTFR, by assessing carbohydrate and fat metabolism, as well as related hormones. Better understanding of the influence of estrogen on these factors may have critical implications for understanding the effectiveness of food restriction for weight loss (ie. dieting) and, perhaps, for success in maintaining body weight over the long term.

CHAPTER II

LITERATURE REVIEW

Obesity is a major problem in the US (61, 62), especially for women and, in particular, after menopause when body weight increases (99). Women diet to lose weight by decreasing the number of meals or the amount of calories consumed, but with variable effectiveness. Thus, animal models of obesity and feeding are critical to greater understanding of the controls of eating. A wealth of previous studies, many in rats, have shown that feeding is influenced by hunger, satiety, and physiological mechanisms that link eating with the availability of calories, and a with stable body weight (1, 75, 127, 132, 144, 175). In short, the controls of food intake and body weight are complex processes subject to inhibitory and stimulatory signals including gastric, hormonal, and metabolic signals, *or* a combination of these signals.

In 1925, Wang reported changes in body weight and food intake during the ovarian cycle of rats (170) suggesting that ovarian hormones have a role in the control of feeding and body weight. Consistent with this finding, body weight increases after menopause in humans (100) and after OVX in rats (8), further supporting the involvement of estrogen and/or progesterone in weight control. Subsequent studies showing that estrogen treatment inhibits food intake and body weight gain in *ad libitum* fed OVX rats (48), point more conclusively to estrogen in the control of food intake and body weight. This effect may be due to estrogen-mediated changes in *levels* of

any of the inhibitory or excitatory signals that control feeding and body weight. Alternatively, estrogen may alter *sensitivity* to such signals, via interactions with the CNS.

CNS, feeding and body weight

Many studies have implicated a number of CNS areas in the regulation of food intake and body weight (15, 16, 113, 154, 158). Major hypothalamic regions implicated include the arcuate nucleus (ARC), from where neurons project anteriorly to other CNS areas involved in the control of feeding and body weight, including the paraventricular nucleus (PVN) and the lateral hypothalamic area (LHA). Other hypothalamic regions implicated in regulating food intake include the ventromedial nucleus (VMN) and the dorsomedial nucleus (DMN). The PVN and the LHA are part of central autonomic circuits that project to hindbrain areas involved in the control of feeding such as the NTS. At the same time, signals arising from the liver, gastrointestinal tract, and gut peptides are transmitted through the vagus nerve to the NTS and the adjacent area postrema (AP). These signals are integrated with descending hypothalamic input (154). Finally, circulating hormones and metabolites are detected by circumventricular organs (CVOs) such as the hindbrain AP and the forebrain subfornical area and organum vasculosum laminae terminalis (OVLT).

In male rats, c-FOS expression (a marker of neural activation) is increased in the CNS after ingestion of meals (141). The NTS is the hindbrain site at which numerous signals related to the control of feeding including leptin, serotonin (5HT), and glucagon-like peptide-1 (GLP-1) (5, 6, 35) are processed. In addition, the anorexigenic neuropeptide oxytocin (OT) regulates gastric motility and activity of OT neurons is increased in response to stomach distention or to increased osmolality (120). OT neurons in the hypothalamus send projections to two major brainstem sites including the dorsal motor nucleus of the vagus (DMV), which provides efferent and afferent vagal innervation of the stomach, and the AP, a CVO which lacks a blood brain barrier and monitors

peripheral signals. Thus, these interconnected areas play an important role in the detection of feeding-related information, which includes GI motility, plasma hormones, and plasma metabolites (74, 107).

Estrogen, estrogen receptor distribution, and regulation of food intake and body weight

Estrogens act through two types of estrogen receptors (ER): classic nuclear receptors (ERα and ERβ) and novel cell membrane receptors (GPR30, ER-X and ERα36). Both types of estrogen receptors are expressed both peripherally and in the CNS, with cell- and tissue-specific distributions. For example, estrogens from the ovary, interacting with nuclear estrogen receptors, play major roles in the regulation of the reproductive system, such as pubertal onset, fertility, and the estrous cycling, while estrogens in the CNS protect against insult-induced neuronal damage via both nuclear and cell membrane receptors (177, 178).

In the CNS, both ERα and ERβ are widely distributed and are expressed in both neuronal and non-neuronal cell types (89, 109, 131). Although overlapping expression of ERα and ERβ has been demonstrated in some brain regions, the levels of expression, as well as the distribution patterns of ERα and ERβ, are largely distinct. The distribution of ERα and ERβ is related to receptor function. ERα is the most abundant subtype in the hypothalamus and amygdala, which are key regions involved with neuroendocrine function, and estrogen helps to regulate the autonomic nervous system and emotional reactions. ERβ levels are greatest in the hippocampus, and cerebral cortex, and are least in the hypothalamus (121, 156). Studies using ERα and ERβ knockout mice have shown that ERα, but not ERβ, is required for both negative- and positivefeedback regulation of gonadotropin-releasing hormone (GnRH) neuron activity by estrogen (26). In contrast, ERβ is primarily involved in mood and cognitive activities (28). The functional significance of the dual

expression of both ERs within the same hypothalamic area remains unknown; moreover, it is possible that a given ER plays different roles in gene regulation (125, 126).

A specific role for ERα in the control of body weight and food intake also is supported by studies with transgenic mice with null mutations affecting estrogen signaling via ERα (69). These mice had excess body weight and body fat, as well as decreased energy expenditure, a series of effects resembling metabolic syndrome. In addition, estradiol treatment of these mice, which were OVX after puberty, failed to reduce daily food intake, weight gain or fat gain as it did in wild type mice (65). Microinjections of low doses of estradiol directly into the brain were shown to inhibit food intake (30, 122). Knockdown of VMH ERα results in obesity due to an anabolic process, with changes in energy expenditure primarily mediating the weight gain (115).

Other CNS areas have been investigated. In OVX female rats, c-FOS expression increased in the NTS after eating a satiating meal, and in experiments where the amount of food eaten was constant for EB- and OIL-treated groups, estrogen increases c-FOS labeling further (50). However, experiments examining c-FOS labeling after treatment with the 'satiety factor', cholecystokinin (CCK) in EB-treated rats report increases or decreases in c-FOS in the NTS (51, 60).

A role for the NTS was demonstrated by Thammacharoen and colleagues who showed that OVX rats implanted with EB in the hindbrain just caudal to the AP lost body weight (162). In fact food intake in these rats was decreased from control animals during days three and four after surgery, a response time consistent with classical ER activation. Taken together, these observations suggest that the binding of estradiol to ERα in the hypothalamus, or elsewhere in the CNS, may represent a mechanism by which estradiol regulates food intake, body weight, and possibly body fat distribution.

Gastric Mechanisms, Feeding and Body Weight

Because meals end long before substantial digestion and absorption occur (136), gastric factors likely serve as satiation signals. One obvious possibility is gastric distension, and meals do end when the stomach is distended (136). The stomach wall has many stretch receptors, the activity of which increases in proportion to the volume in the stomach (136). Those signals are transmitted via the vagus nerve to the NTS and adjacent AP in the brain stem (136), from which the information is conducted to the hypothalamus and then to the cortex, where gastric distension is then perceived (136).

Another gastric factor is gastric motility, especially as it relates to prandial periods. When ingested food enters the stomach, the motor pattern changes substantially: the proximal stomach relaxes and initially serves as a reservoir, then by tonic contraction, moves the food distally. The distal stomach in turn mixes and grinds the food by regular and powerful peristaltic contractions. In short, the immediate postprandial processes initially allow for large amounts of food to be taken in while the proximal stomach is relaxed, generating distension signals that play a major role in satiation. The subsequent mechanical aspect of digestion occurs more distally and is followed by tonic and peristaltic contractions that allow a controlled flow of food into the duodenum. Absorption of nutrients from the duodenum and further along the GI tract also provide 'satiety' signals. In the period between meals, proximal stomach muscle tone is high while the distal stomach is engaged in a recurrent contraction pattern known as the migrating motor complex (MMC), which serves to clear the stomach during fasting between meals (78). However, extended dietary restriction (e.g., 2 hours per day for 4 months) delays gastric emptying in rats; emptying returns to normal after three months of free feeding. However, the mechanism(s) of these changes is unknown (143).

Little work has focused on estrogen effects on gastric factors. In experiments including *ad libitum* fed diestrus rats or OVX rats treated with estradiol, estradiol with or without progesterone inhibited gastric emptying, while progesterone alone enhanced gastric emptying (25). The effect of estrogen to decrease gastric motility may involve increasing CCK secretion and CCK receptors on stomach (176).

Hormonal and Peptide Mechanisms, Feeding and Body Weight

Insulin

Hormones provide important gluco-regulatory signals to the brain. After Kennedy (1953) hypothesized that fat stores produced a hormone that functioned as a negative feedback control for adiposity (84), one early suggestion was that insulin was the hormone involved (13). Insulin increases during meals and other periods of positive energy balance and decreases during fasting and other periods of negative energy balance via substrate availability. During meal anticipation, the smell and then the taste of food stimulates insulin secretion. The process that underlies anticipatory insulin release involves input from the forebrain via the hypothalamus to the DMV in the caudal brainstem, which is then relayed to the pancreas by way of cholinergic efferent nerves of the vagus (116). This process, along with gastrointestinal signals, insures that the amount of insulin secreted is appropriate to efficiently use and store the ingested food.

Manipulation of gonadal steroid hormone levels can influence CNS sensitivity to insulin (30). This interaction indicates that the relative amounts of androgen and estradiol are key determinants of CNS sensitivity to the catabolic actions of insulin: proportionally less estrogen, favors insulin sensitivity (30).

CCK

Meal size and cessation of eating are controlled, at least in part, by hormonal signals from the gut such as cholesystokinin (CCK) (10). CCK, which is secreted during meals in response to food, especially lipids, acts on CCK receptors located on vagal afferent fibers that carry gastric stretch signals from the pyloric region of the stomach to the brainstem as well as on CCK receptors in the AP (137). Thus, small amounts of CCK can act synergistically with gastric distension to inhibit feeding via peripheral and central effects.

Estrogen potentiates CCK-induced reduction of feeding in *ad libitum* fed rats (8-10). Importantly, at doses of estrogen similar to those used in experiments examining estrogen effects on feeding (9, 50, 64), plasma CCK was elevated (176) suggesting that changes in CCK may contribute to reduced feeding and therefore body weight in *ad libitum* fed rats.

Leptin

Leptin is secreted as an adiposity signal from the peripheral adipose tissue in direct proportion to body fat mass. Leptin is detected by CNS receptors located in hypothalamic and brainstem areas involved with the control of food intake (154, 174), and at which ER also are found (21, 30, 43). This leptin circuit is fairly well defined. Leptin influences not only food intake, but also sympathetic nerve activity via central actions. In food restricted animals, leptin decreases, and animals readily eat when food becomes available (92). Leptin not only suppresses food intake, but also enhances energy expenditure to reduce fat depots (171). When male rats were placed on a restricted feeding schedule, leptin rhythms were influenced by feeding and reflected levels of corticosterone and thyroxine (3). These findings suggest that leptin may have neuroendocrine actions secondary to those related to energy homeostasis (2). In agreement with these studies, fasting decreased leptin and increased in corticosterone in adult mice (3).

The stomach is an additional source of leptin (11, 27, 157) that may influence feeding by another mechanism. Specifically, duodenal infusion of leptin in rats increased plasma CCK to levels comparable to those induced by feeding (68), demonstrating that leptin, acts to reduce feeding via lumenal leptin receptors (68). Thus, under physiological conditions, leptin may potentiate CCK actions by stimulating its secretion and thereby decrease food intake.

Leptin levels are greater in females compared with males, even before puberty, and these levels are independent of differences in body adiposity (42, 155). After puberty, estrogen and testosterone further modulate leptin synthesis and secretion via sex steroid receptor-dependent transcriptional mechanisms (103). Not surprisingly then, manipulation of gonadal steroid hormones can influence sensitivity to leptin and also affect body fat distribution (30).

Ghrelin

Ghrelin is the only known circulating peptide hormone that stimulates food intake (36). In individuals under restricted feeding schedules, ghrelin levels increase approximately 1hr prior to food presentation, and this preprandial increase is associated with increased locomotor activity in anticipation of a scheduled meal (47). Ghrelin is synthesized and released primarily by endocrine cells in the stomach. The increases in plasma concentrations of ghrelin before meals and rapid decreases after meals, in both humans and rats (38, 164), are consistent with a hungerinducing action. Moreover, basal ghrelin levels are decreased in obese humans and increased by weight loss (38, 165). However, a role for ghrelin in control of food intake and body weight remains unclear, as plasma ghrelin concentration in humans also increases during the evening and decreases in the early morning hours in the absence of eating (38).

Estrogen decreases the potency of ghrelin, suggesting that weight gain after OVX may be ghrelin mediated (31). Specifically, in OVX rats which received peripheral or central doses of

ghrelin that did not stimulate eating (31), EB treatment decreased the orexigenic actions of ghrelin. Food restriction increases serum ghrelin but decreases both gastric and hypothalamic ghrelin in female rats (4). These findings led investigators to conclude that ghrelin may contribute to the suppression of the female reproductive axis during negative energy balance and to propose that ghrelin may be a link between body weight homeostasis and reproductive function (4).

Central peptides and neurotransmitters

Neuorpeptide Y (NPY)

Hypothalamic NPY is an important central regulator of energy homeostasis (29). It is a potent orexigen, increasing feeding behavior in fed and fasted animals (81). Since estrogen acts via ERs in the hypothalamus to reduce feeding (169), estrogen's anorectic effects may be mediated by decreasing NPY expression or release (18). Though it remains unclear whether estrogen directly affects NPY neurons, colocalization of ER and NPY immunoreactivity in ARC neurons has been reported (152) and estrogen treatment decreased NPY release into the PVN of OVX rats (18).

Melanin-concentrating hormone (MCH)

MCH has been recognized as an important regulator of energy homeostasis (129) since its discovery in hypothalamic neurons (166). Central administration of MCH promotes feeding and the *Mch* gene is upregulated by fasting (130). Santollo, et al. observed a greater increase in food intake, meal size, and water intake following MCH treatment in male rats than in estradioltreated OVX rats. They also reported that greater MCH doses were necessary to increase food intake in estradiol-treated OVX rats (149), leading them to suggest that estradiol reduces MCH sensitivity in female rats by decreasing either the *number* or the *binding affinity* of MCH-1 receptors.

Corticosterone

Adrenal steroids suppress growth of experimental animals, inhibit wound healing, cause involution of lymphoid tissue, impair bone growth and produce atrophy of skin and subcutaneous tissue (reviewed in (55)). In addition, the hormone corticosterone is a potent effector of carbohydrate, protein and fat metabolism. Corticosteroid action on carbohydrate metabolism involves increases in gluconeogenesis as demonstrated in adrenalectomized, fasted rats (98). Studies of glucose synthesis from 3-carbon precursors provided additional evidence for increased gluconeogenesis (95). It has been postulated that adrenal steroids promote carbohydrate synthesis because they stimulate activity of enzymes in the gluconeogenic pathway (110). However, rather than being a primary effect of corticosteroids, the alteration of such enzyme activity may represent a secondary effect that results from the increased activity in the gluconeogenic pathway. Another potential estrogen effect of increased enzyme activity is activation of the type *a* phosphatase that activates glycogen synthetase (110), increasing glycogen deposition.

The first demonstration of the important influence of corticosteroids on protein metabolism came from Long and colleagues in 1940, (98) but many investigations since have shown that adrenal cortical steroids promote protein catabolism and interfere with protein synthesis (reviewed in (55)). Corticosteroids increase levels of free amino acids in muscle and reduce capacity of muscle to concentrate amino acids, seemingly due to inhibition of protein synthesis (114). It is generally recognized that muscle is the chief site of protein breakdown and source of amino acids following steroid administration. In contrast, liver amino acid 'trapping' is stimulated by corticosteroids and the increase in liver protein synthesis resulting from steroids is accompanied by an increase in liver tissue RNA content (85).

Inhibition of lipogenesis by adrenal steroids has been observed by many investigators (55, 101). Although hormonal regulation of lipid mobilization results primarily from controlling the rate of triglyceride hydrolysis in adipose tissue by catecholamines, growth hormone (GH) and glucocorticoids can act together *in vivo* to increase fatty acid release from adipose tissue (87). In fact, corticosteroids do not alter lipolysis in adipose in the absence of GH. In contrast, corticosteroids alone exert an important lipolytic action in liver (87).

In men, estradiol increased cortisol binding protein, but did not increase basal cortisol secretion (83). In post-menopausal women receiving estrogen replacement therapy, free and bound cortisol were increased compared to controls receiving progesterone with estrogen (54). Subcutaneous injection of estradiol increased corticosterone production in the adrenal zona fasciculata-reticularis (ZFR) cells from OVX rats (97). Increased corticosterone with estrogen may involve the hypothalamic-pituitary-adrenal axis.

Metabolic Mechanisms, Food Intake and Body Weight

All cells of the body depend on continuous supplies of metabolic fuels to support ongoing activity. Delivery, storage and mobilization of lipid and carbohydrate metabolites are regulated and the control systems involve several hormones as well as the sympathetic and parasympathetic nervous systems. Fuels enter the circulation from the small intestine during the prandial period and from storage depots during fasting. The availability of fuels to supply tissues is primarily controlled by the liver, the key organ in energy management, and by the hormone insulin (173). Hepatic function and insulin secretion are, in turn controlled by the autonomic nervous system. In *ad libitum* fed rats, lipogenesis (which also occurs in adipose tissue) and glycogen formation occur in the liver during the prandial period, and glycogenolysis, ketogenesis

and gluconeogenesis occur during the fasting period. Plasma glucose levels decrease rapidly immediately before eating in food restricted rats (96). A single 24 hour fast (or insulin administration) produces more rapid depletion of liver glycogen than does chronic food restriction. Glycogen levels are increased or unchanged during food restriction (71), whereas lipogenesis in liver and adipose tissue are increased compared to *ad libitum* fed rats (71, 96).

Carbohydrates and metabolism

Circulating levels of glucose are controlled by two hormones, insulin and glucagon. In response to elevated glucose levels, proinsulin is released from pancreatic β cells and converted to the active form. When insulin binds to the insulin receptor, a signaling phosphorylation cascade starts, which leads to translocation of vesicles containing glucose transporter 4 (GLUT4) to the plasma membrane facilitating glucose entry into the cells (179). Insulin, as a result, stimulates the uptake and storage of glucose as free fatty acids (FFA) in skeletal muscles and adipose tissue or as glycogen through glycogenesis in the liver. Glucagon, on the other hand facilitates glucose-1-phosphate release from glycogen polymers, through the action of G-protein receptors on the plasma membrane activating glycogen phosphorylase.

The most important factors influencing glucose short-term fluxes are the hormones (insulin, glucagon and catecholamines), the sympathetic nervous system, and the concentration of other substrates (FFA). Long-term control involves other hormones (cortisol and growth hormone (GH)), diet composition, physical fitness and changes in the sensitivity to the various hormones. For example, cortisol, GH and catecholamines affect glucose homeostasis by changing insulin sensitivity and changing availability of alternate fuels. While FFA are the main fuel for most organs of the body, glucose is the metabolic fuel of choice for the CNS under physiologic conditions. However the CNS cannot synthesize glucose or store it as glycogen in amounts

sufficient for more than a few minutes supply. Thus, CNS is dependent on a continuous supply of glucose from plasma.

Carbohydrate catabolism involves glycolysis and Kreb's cycle, ultimately producing ATP through the electron transport system. Excess glucose from meals, along with glucose produced by gluconeogenesis, is stored in the form of glycogen in the liver and catabolically reduced to glucose for metabolic fuel during fasting. When fasting is prolonged, the proportion of glucose produced by gluconeogenesis is increased, and contribution of hepatic glycogen stores to the glucose pool decreases, while the contribution of the kidney glycogen stores increases (66). After an overnight fast, there is no net storage of glucose and all of the glucose taken in by the tissues is completely oxidized (or converted to lactate in the absence of oxygen). The main regulator of insulin secretion is plasma glucose levels; insulin increases several fold after a meal and decreases to minimal levels during fasting.

Glucose exerts vagally-mediated effects upon gastric motility and emptying which in part, stabilizes large fluctuations in blood glucose levels following meal ingestion (59, 72, 77, 102, 139). An increase in gastric motility in response to hypoglycemia accelerates nutrient delivery to the intestine, which allows increased absorption, and thereby re-establishes physiological plasma glucose levels (59). In contrast, decreased gastric motility in response to hyperglycemia delays gastric emptying and reduces additional glucose absorption, thereby preventing prolonged, and damaging elevations in glucose levels (59). Protein also regulates blood glucose by stimulating insulin and glucagon (147). In fact oral protein is the most effective macronutrient to modulated postprandial glycemia.

In addition, glucose directly alters the activity of enteric nervous system neurons independent of the CNS. Intra-intestinal infusions of glucose activate sensory neurons in the myenteric and submucosal plexuses of the upper small intestine (153, 167). Glucose also

modulates the response of enteric neurons to other GI neurohormones such as CCK and serotonin (145). Further, glucose appears to decrease gastric motility and delay gastric emptying via paracrine mechanisms. For example, glucose within the lumen of the intestine induces the release of neurohormones from enteroendocrine cells, including serotonin from enterochromaffin cells within the proximal intestine, and glucagon-like peptide-1 (GLP-1) from L-cells in the distal intestine. These neurohormones activate receptors (5-HT3 and GLP-1 receptors, respectively) on peripheral vagal afferent nerve terminals and the resulting excitatory signals are transmitted centrally (67, 133-135, 138). These sensory signals activate second order neurons within the NTS and, after integration, the resulting vagal motor response produces gastric relaxation and delayed emptying (59, 138, 180).

Studies of humans and mice show relationships between estrogen and the regulation of glucose homeostasis (90, 104). Estrogen increases glucose uptake in skeletal muscle and adipose tissue and decreases gluconeogenesis in liver (58). Hormone replacement therapy (HRT) has been shown to improve insulin sensitivity and to lower blood glucose in healthy postmenopausal women and to reduce the incidence of type 2 diabetes in postmenopausal women with coronary heart diseases (34, 82). Importantly, men with a deficiency in the enzyme that aromatizes testosterone to estrogen and therefore cannot synthesize estrogen, have impaired glucose metabolism and increased insulin resistance (112). Female mice with intact ovaries are protected against hyperglycemia whereas aromatase knockout mice are insulin resistant (80). ER has been shown to be involved in the maintenance of glucose metabolism in several tissues including liver, skeletal muscle, adipose tissue, pancreatic β cells, and CNS (7). Estrogens are also known to regulate pancreatic β cell function through an ERα-dependent mechanism. One study in mice suggests that long-term estrogen exposure increases insulin levels, insulin, gene expression, and insulin release, without changing β cell mass (7). Estrogen-dependent insulin release in cultured

pancreatic islets was reduced in ERα-deficient mice, when compared with islets derived from either ER□-deficient or wild type mice (7). However, ERβ-deficient mice show mild pancreatic islet hyperplasia with delayed initiation of insulin resistance (12) suggesting that ERβ interrupts pancreatic β cell functioning.

Lipids and metabolism

FFA are the main fuel for most organs of the body and they are released from triglycerides by hydrolysis of ester bonds. In the duodenum, the presence of lipids increases the secretion of CCK which causes the release of bile salts from the gall bladder and release of pancreatic lipases to further digest lipids. Short-chain FFA are absorbed via blood capillaries and eventually empty into the portal vein where they are transported to the liver and other organs for energy production. Longer-chain FFA, are re-esterified and enter the blood via the lymph vessels as chylomicrons. Triglycerides in chylomicrons are cleared by lipoprotein lipase (LPL) at the capillary endothelium and can be stored as fat in adipose tissue. During fasting elevated glucagon, and during exercise, elevated epinephrine, activate hormone-sensitive lipase (HSL) to hydrolyze triglycerides to glycerol and FFA. The FFA then are used as fuel in most tissues, except the CNS. During fasting or exercise LPL, fatty acid synthesis and triglyceride synthesis are inhibited. In the fed state, insulin inhibits HSL and no FFA are released from adipose tissue. Insulin also stimulates glycolysis and inhibits gluconeogenesis in the liver as well as stimulating fatty acid and triglyceride synthesis in the liver.

The role of specific fatty acids and lipids in body weight control focuses on their utility in promoting satiety with respect to energy intake, body composition, energy expenditure, fat oxidation and energy efficiency. The presence of food, particularly fat (20), or leptin (68) in the upper small intestine stimulates release of CCK, which has regulatory roles in bile secretion, gastric

emptying and exocrine pancreas activity, in addition to vagally-mediated effects to inhibit eating and terminate meals (41).

The delaying effect of lipid on gastric emptying is increased in the elderly, and the administration of lipase accelerates the emptying of lipid from the stomach (117). In men, it is possible to delay gastric emptying and increase satiety using fat emulsions that have been stabilized against the acidic gastric environment (106). In lean men, it has been shown that lipid placed intraduodenally modulated gastrointestinal motility and hormone release and suppressed energy intake more than did glucose placed intraduodenally. Despite differing effects on gut function, both lipid and protein in the duodenum produced (44) comparable reductions in energy intake.

Adipose tissue and liver are the most important tissues for the influence of EB on lipid metabolism. Estrogens influence adipose tissue cells and liver cells by endocrine and paracrine mechanisms and are also produced in these cells from androgens found there. Moreover, estrogen can be stored as esters with long-chain fatty acids in adipocytes (161). ERs are present in adipocytes (44, 161) and hepatocytes (161), but their density is much lower than that in gonads. On the cellular level, estrogens regulate mRNA production for particular proteins, among which are the proteins involved in lipid metabolism. In adipose tissue, EB has a direct effect on LPL and HSL (161). Specifically, LPL synthesis is more rapid, while the synthesis of HSL is delayed in the presence of estrogen. Finally, indirect action of estrogens on adipose tissue is connected with the release of other hormones which increase HSL activity, including catecholamines, GH and glucagon (161).

Clearly, estrogen levels in reproductively intact females are high enough to change lipid metabolism. *Accumulation* of adipose tissue is sexually dimorphic (140) with females having a

higher percentage of body fat than males. Fat *distribution* is also different with females accumulating more subcutaneous fat and males accumulating more visceral fat (19) (34) (118). Estrogen deficiency or the decline in estrogen levels after menopause can cause dysregulation of metabolism. The onset of menopause is associated with several metabolic changes and postmenopausal women fall into the same risk category as men for development of atherosclerosis and myocardial infarction (23). Other changes associated with low estrogen levels are insulin resistance, impaired glucose metabolism, increased hepatic gluconeogenesis with subsequent glucose secretion, and increased levels of inflammatory markers (58).

Finally, Clegg et al. (30) report that estrogen regulates body fat distribution, interacts with the adiposity signal leptin, and enhances leptin's central action to alter sympathetic outflow to the visceral fat. This sympathetic action facilitates fat mobilization in the visceral depot and fat deposition in the subcutaneous depot. Estrogen regulates body adiposity and fat distribution through its receptors ERα and ERβ (91). However only ERα has been reported to have a major influence on energy homeostasis (69). Heine et al. (69) reported that male and female mice with total body deletion of ERα, have increased adiposity in both male and female mice, suggesting an important role for this estrogen receptor in the regulation of body weight and adiposity.

Targeted Temporal Food Restriction (TTFR)

Much of the advances in understanding integrated physiological, hormonal, metabolic and central control of food intake and body weight, and EB effects on these processes, are the result of studies conducted during *ad libitum* feeding conditions. However, many obese people restrict their eating and consume most of their food during the evening, when activity is less, eating little or no breakfast or lunch (14, 46, 63). Substantial alterations to integrated control processes for feeding and body weight may occur during TTFR.

TTFR was used to examine changes in metabolism and the regulating hormones in male rats that were given more food than they could consume in the two hour period allowed for eating (70). These rats lost about twenty percent of their starting weight within the first few days which then increased gradually to return to the starting weight over the next three weeks. Food consumption in the two hour period also increased during TTFR, ultimately returning to 95 percent of the food consumed during the *ad libitum* period. Importantly, the large load of nutrients taken in during TTFR was not stored as glycogen, but entered the system as newly synthesized lipids. This effect was proposed to be due to adaptation to the feeding program, such that male rats metabolized fat as their major energy source and limited glucose utilization to the small amounts required by the central nervous system (70). Lima, et al (96) also employed TTFR in male rats but gave 2 hours of access to chow either in the morning or at night. In these studies, food intake did not increase to levels approximating those during *ad libitum* conditions with day or nighttime feeding, nor did body weight increase during the experiment. In addition, their results showed that when food availability was restricted to short periods of time as with TTFR gastric emptying is much slower than in *ad libitum* feeding conditions. Lima and colleagues also proposed an adaptive hypertrophy in gastric mucosa during TTFR that allows increased absorption. Based on these experiments, rats allowed to eat for only a short period each day, as with TTFR, may have more efficient nutrient uptake and utilization (32). With prolonged TTFR, changes in gastric function occur along with metabolic changes that increase lipogenesis in liver and fatty tissue (94) and decrease hepatic glycogen depletion (93).

Female rats have been subjected to TTFR in various feeding protocols (feeding for 2-4 hr one or more times per day) (123, 128, 160, 163). These studies focused on the effects on lipogenesis in brown adipose tissue, glycogen and glucose synchronization to the phases of the circadian cycle during TTFR, and the advantage of TTFR-induced diminished insulin release in the

progression of breast tumors. However, estrogen effects on the CNS or metabolic factors that affect body weight and food intake during TTFR have not been investigated in the female rat.

In this dissertation research, I use OVX rats to model the restricted eating schedule of some obese people, who eat little breakfast and lunch but eat most calories in the evening at the low energy time of day, by giving the rats food access two hours during the day when their activity is less (i.e. a TTFR protocol). My goal is to study estrogen's effects on the signals that control eating and body weight during TTFR. To accomplish this goal, I have compared OVX rats with and without estrogen treatment during TTFR. I first assessed food intake and body weight during TTFR. Next, I investigated central mechanisms of estrogen effects during TTFR by examining neural interactions in the hindbrain NTS, where vagal afferents from the gastrointestinal tract terminate. Next, I investigated peripheral mechanisms underlying estrogen effects during TTFR, by assessing carbohydrate and fat metabolism, and related hormones. Better understanding of the influence of estrogen on these factors may have critical implications for understanding the effectiveness of food restriction for weight loss (i.e. dieting) and, perhaps, for success in maintaining body weight over the long term.

CHAPTER III

MATERIALS AND METHODS

Animals, Surgeries and Hormone Treatments

Adult female Sprague-Dawley rats (Charles River), three months of age, were housed in individual cages in a temperature controlled (22 ± 2° C) room on a 12:12 light:dark cycle (lights on 0700). All rats were given *ad libitum* access to water and chow (Harlan rodent diet #2018) except as described. Experimental protocols were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and all procedures were approved by the Oklahoma State University Center for Health Sciences Institutional Animal Care and Use Committee. After one week to acclimate to the colony room rats, were bilaterally OVX by a midventral incision under pentobarbital anesthesia (50 mg/kg i.p.; Sigma-Aldrich) and allowed to recover for 7-10 days. After recovery, a regimen of 0.1 ml subcutaneous injections with 17-βestradiol-3-benzoate (EB; 10 µg/0.1 ml sesame oil; Fisher Scientific), or sesame oil vehicle (OIL; 0.1 ml) was initiated, with injections given on two consecutive days at weekly intervals for the duration of the experiment. The experimental protocol is shown schematically in Figure 1.

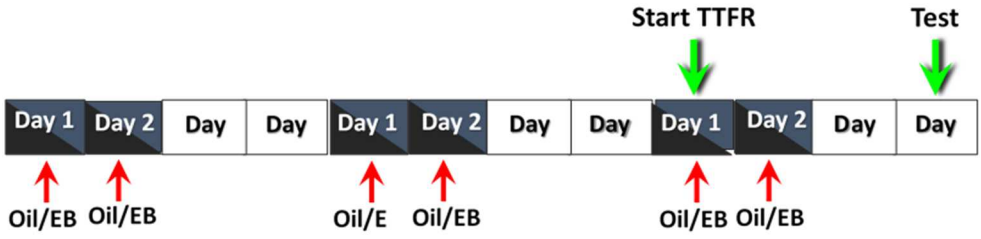


Figure 1: Schedule of EB/OIL injections and experimental protocol.

Feeding Protocol

Rats were weighed each day at 10:00, and chow and water then were removed from the cages. Pre-measured chow was returned to the cages from 13:00 to 15:00, along with water in graduated cylinders fitted with sipper tubes. Water intake was measured after two hours and uneaten chow was collected and weighed to determine intake during this targeted 2-hour intake period.

For the first two weeks (Week 1 and Week 2), pre-weighed chow and water were returned at 15:00. Rats had *ad libitum* access to chow and water overnight and intakes of both were determined the following morning. After this two week period to adapt to the targeted 2-hour intake period, the overnight feeding was discontinued. During the next week (Week 3), access to chow was restricted to the targeted 2-hour period each day (13:00 to 15:00), temporally targeted food restriction (TTFR), though rats continued to have *ad libitum* access to water each night.

Rats were euthanized 48 hours after the second OIL or EB injection on Week 3. On the day of euthanization, some food restricted OVX rats were permitted to eat for 1 hour (FED) and then euthanized one hour later; chow intake during this 1-hour intake test was measured. To control for non-specific effects of TTFR, other food restricted OVX rats were not permitted to eat (FASTED) prior to euthanization on Week 3. Separate groups of OVX rats were maintained on *ad libitum* access to chow until they were euthanized on Week 3 and, thus, served as untreated controls.

CNS IMMUNOLABELING

Perfusion, Brain Extraction and Sectioning

On the day of euthanization on Week 3, rats were deeply anesthetized with pentobarbital (25 mg/rat, i.p.) and then perfused transcardially with 0.15 M NaCl (~300 ml) followed by phosphate buffered 4% paraformaldehyde (~300 ml). Brains were removed, stored in paraformaldehyde overnight at 4° C, and then transferred to 30% sucrose for cryoprotection. Brains were stored at 4° C, until cut into 40-µm sections in a 1-to-3 series using a cryostat (Leica). Sections were stored at -20° C in a cryoprotectant solution (1% polyvinylpyrrolidone, 30% ethylene glycol, 30% sucrose in sodium phosphate; pH 7.2), until processed for ERα or fos immunolabeling.

ERα Immunohistochemistry and Quantification

One series of free-floating hindbrain (NTS) sections was rinsed of cryoprotectant by six 5minute washes with 0.05 M Tris NaCl and then incubated in 0.5% H_2O_2 in 0.05 M Tris NaCl for 30 minutes at room temperature. After six 5-minute washes with 0.05 M Tris NaCl, sections were incubated in 10% normal goat serum (NGS; mixed in 0.5% Triton-X with 0.05 M Tris NaCl) for 1 hour at room temperature. Sections were then transferred to the primary antibody (Millipore, rabbit anti-ERα) diluted 1:10,000 in 2% NGS for one hour at room temperature, followed by 70 hour incubation at 4° C.

Sections were brought to room temperature, rinsed three times for 5 minutes each with 2% NGS and then incubated for ~4 hours in the secondary antibody, (Jackson Immunoresearch goat anti-rabbit IgG - Cy2) diluted 1:300 in 2% NGS. Sections then were rinsed two times with 2% NGS, followed by three rinses with 0.05 M Tris NaCl.

Labeled sections were ordered and mounted on gelatin coated slides and allowed to dry. Sections were dehydrated on the slides in an ascending series of ethanols (70%, 90% and 100%), defatted in xylenes, and coverslips then were attached with Cytoseal (Thermo Scientific). Slides

were examined at 10X magnification with a Nikon Eclipse 80i microscope fitted with FITC and rhodamine filters and NIS Elements software. Sections containing the NTS were identified using the atlas of Paxinos and Watson (124). The NTS was outlined caudal to calamus scriptorius (cNTS; -14.30 to -14.60 mm relative to Bregma); and at the middle level subadjacent to the area postrema (midNTS; -13.68 to -14.08 mm relative to Bregma). ERα-labeled neurons were counted in 2-4 sections at each level that were matched between animals. Average numbers of ER□□labeled cells were calculated for each animal at each level and group means for each level were determined for each feeding condition.

Fos Immunolabeling and Quantification

Another series of free-floating hindbrain sections were rinsed of cryoprotectant, incubated in 0.5% H_2O_2, washed with 0.05 M Tris NaCl, and incubated in 10% NGS (mixed in 0.5% Tween 20 with 0.05 M Tris NaCl). Sections then were transferred to the primary antibody (Santa Cruz SC-52, rabbit anti-c-Fos) diluted 1:30,000 in 2% NGS and incubated at room temperature for one hour before overnight incubation (~18 hr) at 4° C.

The following morning, sections were brought to room temperature, rinsed three times for 5 minutes with 2% NGS and then incubated for two hours in the secondary antibody (Vector biotinylated goat anti-rabbit IgG) diluted 1:300 in 2% NGS. Sections then were rinsed two times with 2% NGS, followed by three rinses with 0.05 M Tris NaCl. Labeling was amplified with avidinbiotin (Vector, Vectastain ABC kit) for 90 minutes and sections then were rinsed. Labeling was visualized with nickel-intensified diaminobenzidine (Vector DAB kit) to produce a blue-black nuclear reaction product. The reaction was terminated by multiple rinses with 0.05 M Tris NaCl.

Labeled sections were ordered and mounted on gelatin coated slides, allowed to dry and then dehydrated and defatted as described. Coverslips then were attached and slides were examined using a Nikon Eclipse 80i microscope. Sections containing the cNTS and midNTS were identified and outlined as described. Fos-positive nuclei within the cNTS and midNTS were quantified in 2-3 representative sections from each area, matched between animals. Average numbers of fos-positive nuclei were calculated for the cNTS and for the midNTS for each animal; group means then were determined for each feeding condition.

PERIPHERAL METABOLIC FACTORS

Peripheral Tissue Harvesting:

Rats that were used to examine peripheral tissues were euthanized with CO_2 and decapitated. Trunk blood was collected into Lithium EDTA tubes and centrifuged to obtain plasma which was then frozen in aliquots for later analysis of hormones and fat and carbohydrate metabolites. Uterus, retroperitoneal fat and adrenals were collected and weighed. Abdominal fat and liver samples were collected and quick frozen for further analysis.

Stomachs were removed, blotted and weighed with contents intact to the nearest 0.1 gram. Then they were sliced open from esophageal to pyloric sphincter, contents were removed and the stomachs were rinsed with saline, blotted and weighed again. Differences in weights were expressed as contents in grams.

Plasma Hormone Analysis:

Leptin, insulin, and corticosterone were measured by ELISA according to the manufacturer's instructions. The Millipore leptin assay is a sandwich ELISA based, sequentially, on: 1) binding of leptin in the sample by a pre-titered antiserum and immobilization of the resulting complexes in the wells of a microtiter plate, 2) after washing purified biotinylated detection antibody is allowed to bind to the immobilized leptin, 3) binding of horseradish peroxidase to the immobilized biotinylated antibodies after free detection antibodies are washed off, 4) wash away of free enzyme conjugates, and 5) quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetramethylbenzidine (TMB). The enzyme activity is measured spectrophotometrically by the increased absorbency at 450 nm, corrected from the absorbency at 590nm, after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured leptin in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of rat leptin.

The Millipore insulin assay is also a sandwich ELISA based sequentially, on: 1) capture of insulin molecules from samples to the wells of a microtiter plate coated by pre-titered amount of a monoclonal mouse anti-rat insulin antibodies and the binding of biotinylated polyclonal antibodies to the captured insulin, 2) wash away of unbound materials from samples, 3) binding of horseradish peroxidase to the immobilized biotinylated antibodies, 4) wash away of free enzyme conjugates, and 5) quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate TMB. The enzyme activity is measured spectrophotometrically by the increased absorbency at 450 nm, corrected from the absorbency at 590nm, after acidification of formed products. Since the increase in

absorbency is directly proportional to the amount of captured insulin in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of rat insulin.

The Enzo corticosterone kit is an ELISA assay. The kit uses a polyclonal antibody to corticosterone to bind, in a competitive manner, corticosterone in the standard or sample or an alkaline phosphatase molecule which has corticosterone covalently attached to it. After a simultaneous incubation at room temperature the excess reagents are washed away and substrate is added. After a short incubation time the enzyme reaction is stopped and the yellow color generated is read on a microplate reader at 405 nm. The intensity of the bound yellow color is inversely proportional to the concentration of corticosterone in either standards or samples. The measured optical density is used to calculate the concentration of corticosterone. Plasma samples were diluted sixty-fold with assay buffer before assay.

Plasma Metabolite Analysis (colorimetric):

Glucose, glycogen, free fatty acids (FFA) and triglycerides (TG) were measured with colorimetric kits with the appropriate blanks according to the manufacturer's instructions.

The BioVision glucose colorimetric assay was used to directly measure glucose in one microliter of the plasma samples undiluted. The assay was done in 96-well microtiter plates and read on the microplate reader. Glucose enzyme mix provided, oxidizes glucose specifically, to generate a product with reacts with a dye to generate color at 570 nm. The generated color is directly proportional to the amount of glucose present and the sample concentrations are read from the standard curve prepared.

In the Abcam assay, glycogen is hydrolyzed into glucose, which is oxidized to form an intermediate that reduces a colorless probe to a colored product with strong absorbance at 450 nm. This assay kit can detect less than 4 μg/ml of glycogen in samples.

FFA were measured using the Cayman Chemical company fluorimetric assay kit in 96-well microtiter plates. The assay utilizes a coupled enzymatic reaction to determine FFA concentrations. Acyl CoA synthetase (ACS) first catalyzes fatty acid acylation of coenzyme A. The acyl CoA produced is oxidized by acyl CoA oxidase (ACOD) and generates hydrogen peroxide. Finally, the hydrogen peroxide, in the presence of horseradish peroxidase, reacts with 10-aceyl3,7-dihydroxyphenooxazine (ADHP) in a 1:1 stoichiometry to generate the highly fluorescent product resorufin. Resorufin fluorescence was measured with an excitation wavelength between 530-540 nm and and emission wavelength between 585-595 nm. The plasma sample concentrations were determined using a standard curve.

In the Abcam assay, TG are converted to free fatty acids and glycerol. The glycerol is then oxidized to generate a product which reacts with the probe to generate color (spectrophotometry at λ = 570 nm). The kit can detect 2 pmol-10 nmol (or 2-10000 pM range) of triglyceride in various samples. The kit also detects monoglycerides and diglycerides.

Liver and Abdominal Adipose Triglycerides:

Frozen liver or adipose tissue was placed into 2 ml microcentrifuge tubes, subsequently weighed and homogenized at 5mg/ml in 4% NP 40 using multiple bursts of a sonic homogenizer (set at 3) on ice. The homogenates were centrifuged in the microfuge at 14000 x g for 5 minutes at 4 degrees C. The supernatants were then diluted ten-fold in deionized water and frozen at -20 degrees C until analysis in the colorimetric kit the following day. In the colorimetric Abcam assay,

TG are converted to free fatty acids and glycerol. The glycerol is then oxidized to generate a product which reacts with the probe to generate color (spectrophotometry at λ = 570 nm). The kit can detect 2 pmol-10 nmol (or 2-10000 pM range) of triglyceride in various samples. The kit also detects monoglycerides and diglycerides.

Liver Glycogen:

Frozen liver was placed into 2 ml microcentrifuge tubes, subsequently weighed and homogenized at 5 mg/ml in the glycogen assay buffer from the analysis kit using multiple bursts of a sonic homogenizer (set at 3) on ice. The homogenates were centrifuged in the microfuge at 14000 x g for 5 minutes at 4 degrees C. The supernatants were then analyzed directly in the colorimetric kit the same day. In the Abcam assay, glycogen is hydrolyzed into glucose, which is oxidized to form an intermediate that reduces a colorless probe to a colored product with strong absorbance at 450 nm. This assay kit can detect less than 4 μg/ml of glycogen in samples.

STATISTICS

All data are presented as means ± standard error of the means.

Change in body weight during *ad libitum* conditions was analyzed using two-way ANOVA with hormone (OIL, EB) and Week (1,2,3) as factors. Change in body weight during Week 3 using twoway ANOVA with hormone (OIL, EB) and day (1,2,3) as factors. Food intake on day 4 was analyzed by two-way ANOVA with hormone (OIL, EB) and feeding condition (ad lib, FED). Pair-wise comparisons of significant main effects or interactions were evaluated using Fisher's LSD test. Percent change is defined as metabolite value minus ad lib value divided by ad lib value x 100.

Numbers of ER in the NTS and numbers of fos positive nuclei in the cNTS and midNTS were analyzed using separate two-way ANOVAs with hormone (OIL, EB) and feeding condition (ad lib, fasted, fed) as factors. Pair-wise comparisons of significant main effects or interactions were evaluated using Fisher's LSD test. Additional planned comparisons of specific groups were made using Bonferroni corrections.

Metabolite and hormone data were analyzed by two-way ANOVA with hormone (OIL, EB) and feeding condition (ad libitum, FED, FASTED) as factors. Stomach and adrenal weights were analyzed by two-way ANOVA with hormone (OIL, EB) and feeding condition (ad lib, FED, FASTED) as factors.

CHAPTER IV

RESULTS

As shown in Figure 2, two-way ANOVA revealed that uterine weights were affected by hormone ($F_{(1,28)}$ = 166.06, $p<0.001$) and were less in OIL treated rats. There was no effect of feeding condition and no interaction between hormone and feeding condition.

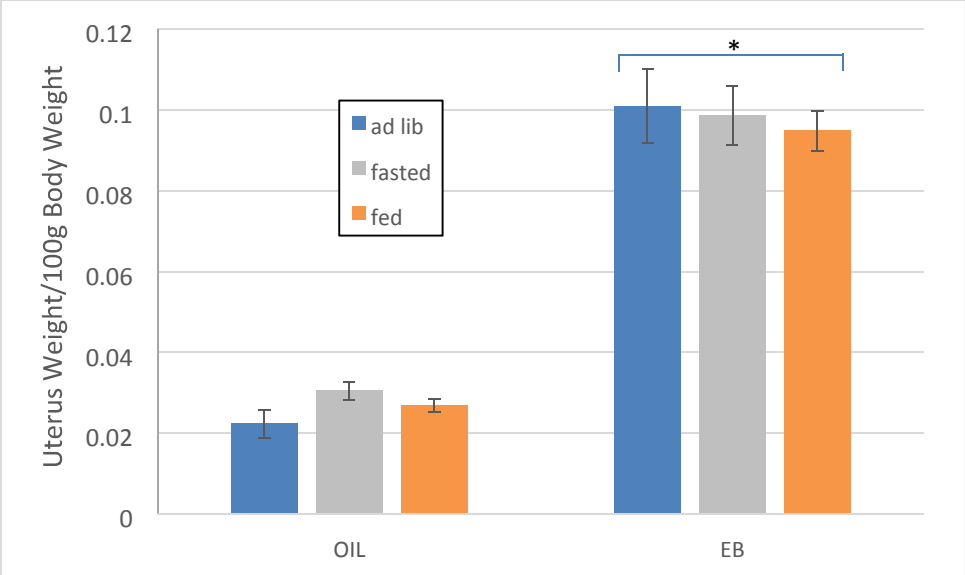


Figure 2: Uterine weights as an indication of estrogen effectiveness. Ovariectomized rats were given subcutaneous injections of 17-β-estradiol-3-benzoate (EB; 10 µg/0.1 ml sesame oil) or the sesame oil vehicle (OIL; 0.1 ml) twice weekly for three weeks. Blue bars denote *ad libitum* fed rats (ad lib; OIL – n=5, EB – n= 6); gray bars denote TTFR rats that were fasted on the test day (fasted; OIL – n= 6, EB – n=6); orange bars denote TTFR rats that were fed on the test day (fed; OIL – n=5, EB – n= 6). Asterisk indicates EB greater than OIL, regardless of feeding condition.

Change in body weight during *ad libitum* condition is shown in Figure 3. Two-way ANOVA revealed that change in body weight was affected by hormone treatment ($F_{(1,77)} = 87.63$, $p<0.001$) with EB-treated rats exhibiting body weight loss compared to the weight gain exhibited by OILtreated rats. There was no effect of week, nor interaction between hormone and week.

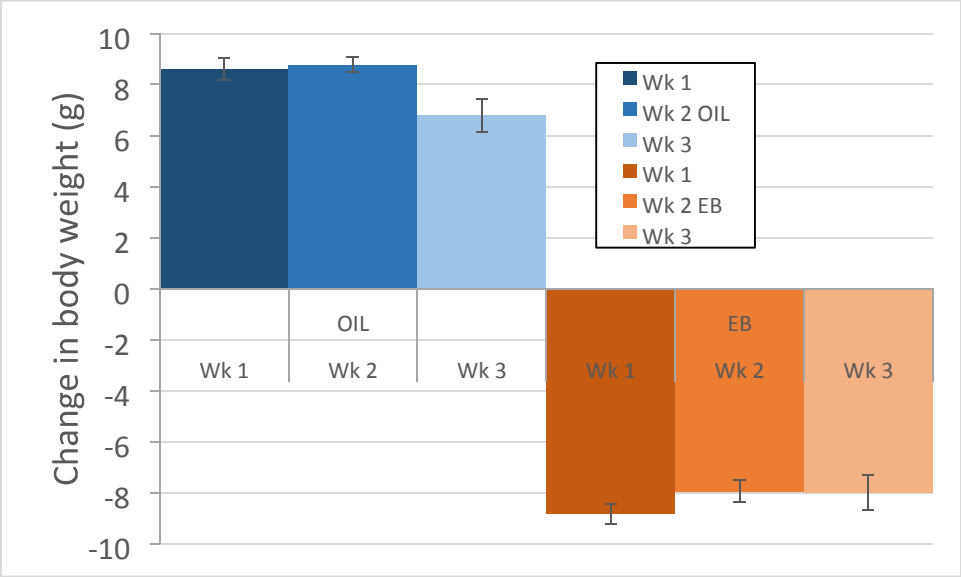


Figure 3: Change in body weight after EB injections twice weekly over three weeks of *ad lib* feeding is a reliable indicator of EB-treatment. OIL: week 1 n=18, week 2 n=18, week 3 n=6; EB: week 1 n=18, week 2 n=18, week 3 n=6.

Figure 4 shows daily body weight during weeks 1-3 in rats fed *ad libitum* throughout (Figure 4a) and in rats in the TTFR condition during week 3 (Figure 4b).

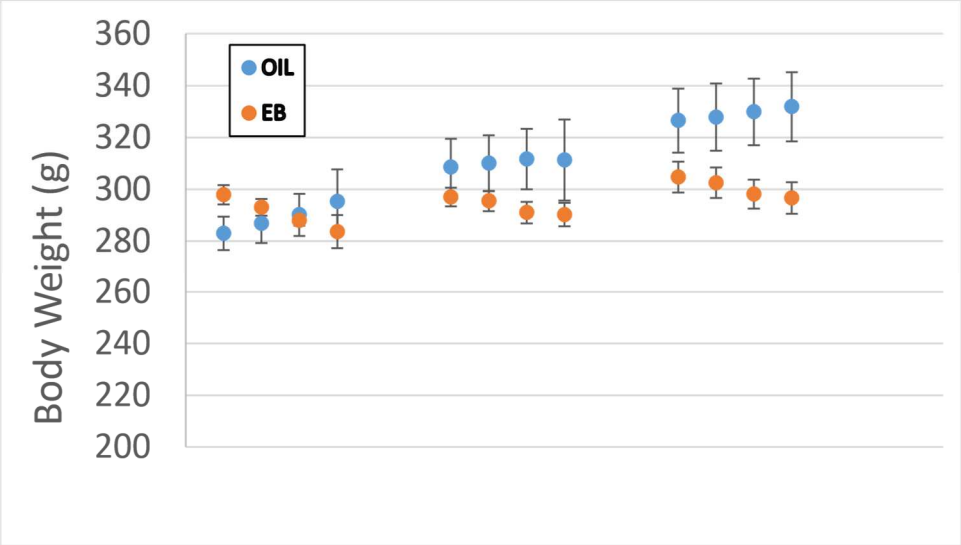


Figure 4a: Body weights of *ad libitum* fed rats over the three weeks of the experiment. Blue filled circles denote OIL treated rats and orange filled circles denote EB-treated rats. OIL, n=5; EB, n=6.

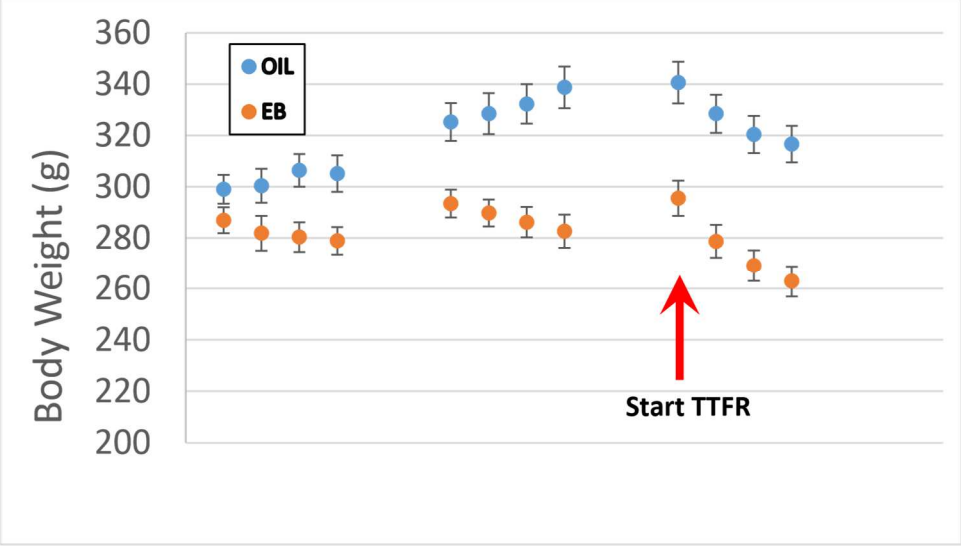


Figure 4b: Body weights of rats that underwent TTFR on week 3, over the three weeks of the experiment. Blue filled circles denote OIL treated rats and orange filled circles denote EBtreated rats. OIL, n=12; EB, n=12

Figure 5 shows the change in body weight each day during week 3. Two-way ANOVA revealed a main effect of day ($F_{(2,66)} = 21.88$, $p<0.001$) and pairwise comparisons showed that, independent of hormone, loss on day 1 was significantly greater than that on both day 2 and day 3 (ps<0.001). Weight loss on day 2 was significantly greater than that on day 3 ($p<0.01$). There was also a main effect of hormone ($F_{(1,66)} = 6.00$, $p<0.05$) with EB-treated rats exhibiting greater weight loss than did OIL-treated rats. There was no interaction between hormone and day.

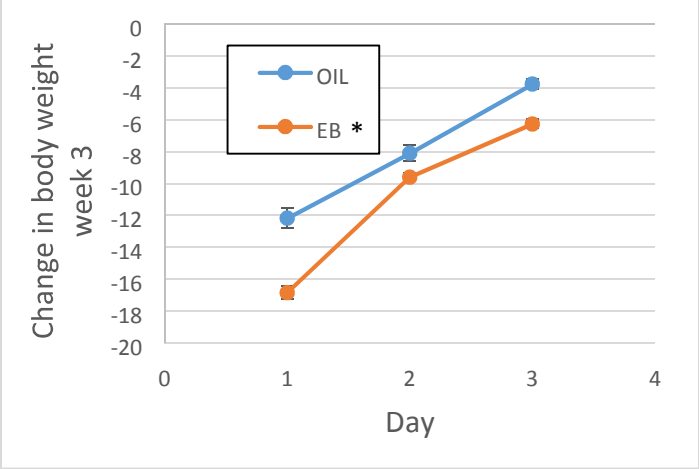


Figure 5: Daily change in body weight during week 3. * = significantly more body weight loss on day 1 than OIL treated controls. OIL n=12, EB n=12.

Food intake on day 4 of week 3 (the day of euthanization), is shown in Figure 6. There was a main effect of feeding condition ($F_{(1,17)} = 58.62$, $p<0.001$), with food intake by the fed group greater than that by the ad lib group, independent of hormone. There was no effect of hormone nor between hormone and feeding condition.

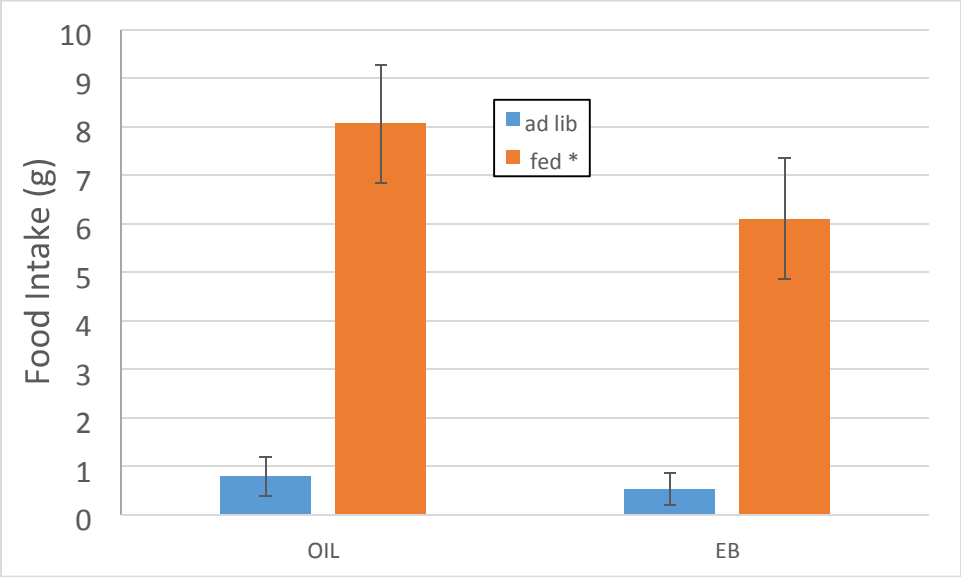


Figure 6: Food intake on day 4 of week 3 TTFR. * = significantly greater than ad lib fed rats. OIL: ad lib n=6; fed n=6 and EB: ad lib n=6; fed n=6.

The weight of stomach contents in the various feeding conditions is shown in Figure 7. Twoway ANOVA revealed a main effect of feeding condition ($F_{(2,30)} = 52.62$, $p<0.001$). Pairwise comparisons showed that, regardless of hormone, stomach contents were significantly greater in the fed groups than in both the ad lib group (which was greater) and fasted group (ps<0.001).

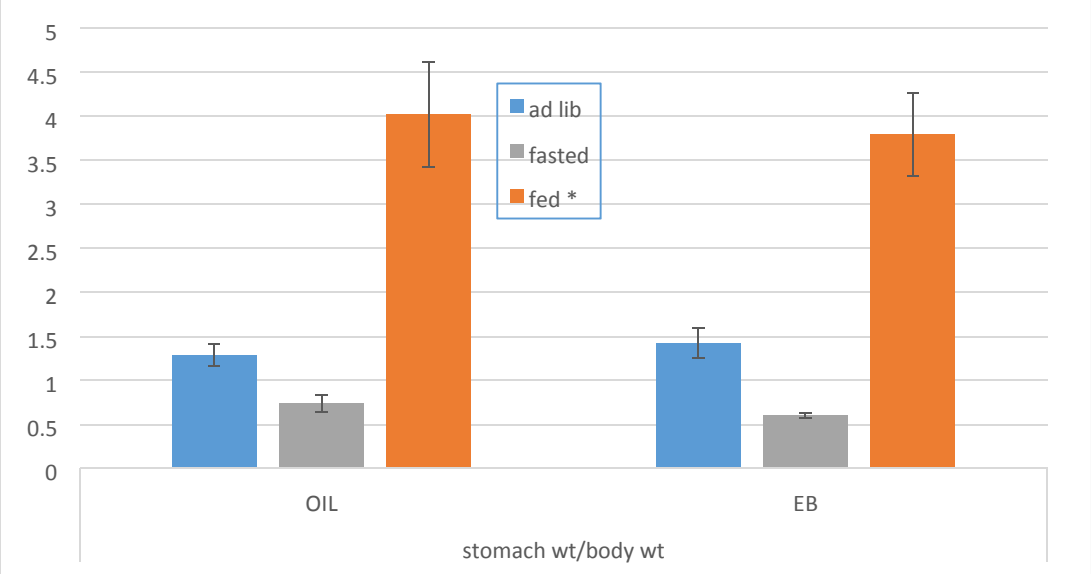
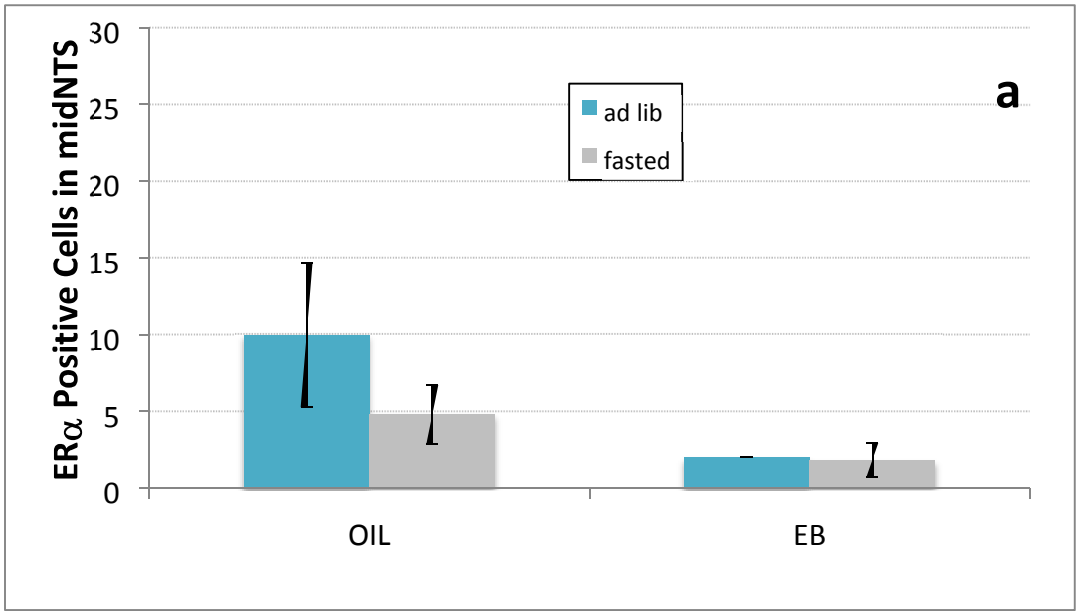


Figure 7: Weight of stomach contents on day 4 of week 3. * = significantly more than ad lib and fasted groups. OIL: ad lib n=6, fasted n=6, fed n=6; EB: ad lib n=6, fasted n=6, fed n=6.

Figure 8 shows quantification of ERα in NTS. Neither feeding condition, hormone treatment, nor the interaction altered numbers of ERα in the midNTS (Figure 8a). Similarly, ERα numbers in the cNTS were not affected by hormone, feeding condition nor the interaction (8b). Representative digital photomicrographs are shown in Figure 8c.



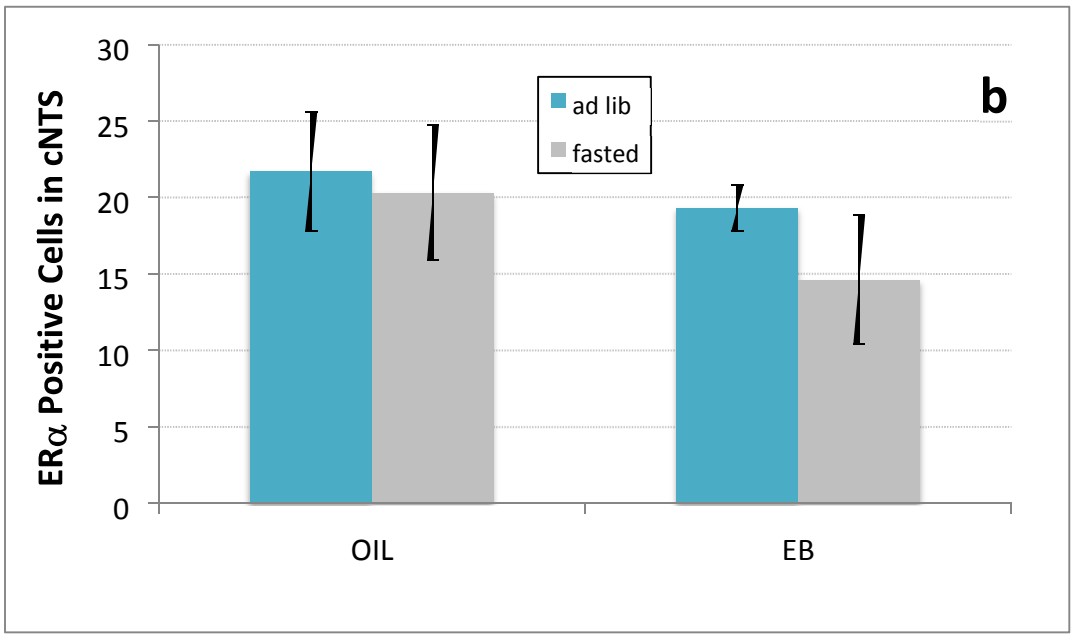


Figure 8: ERα quantification in the nucleus of the solitary tract (NTS). 8a shows the midNTS (OIL: ad lib n=5, fasted n=3; EB: ad lib n=3, fasted n=4) and 8b shows the cNTS (OIL: ad lib n=5, fasted n=3; EB: ad lib n=3, fasted n=4).

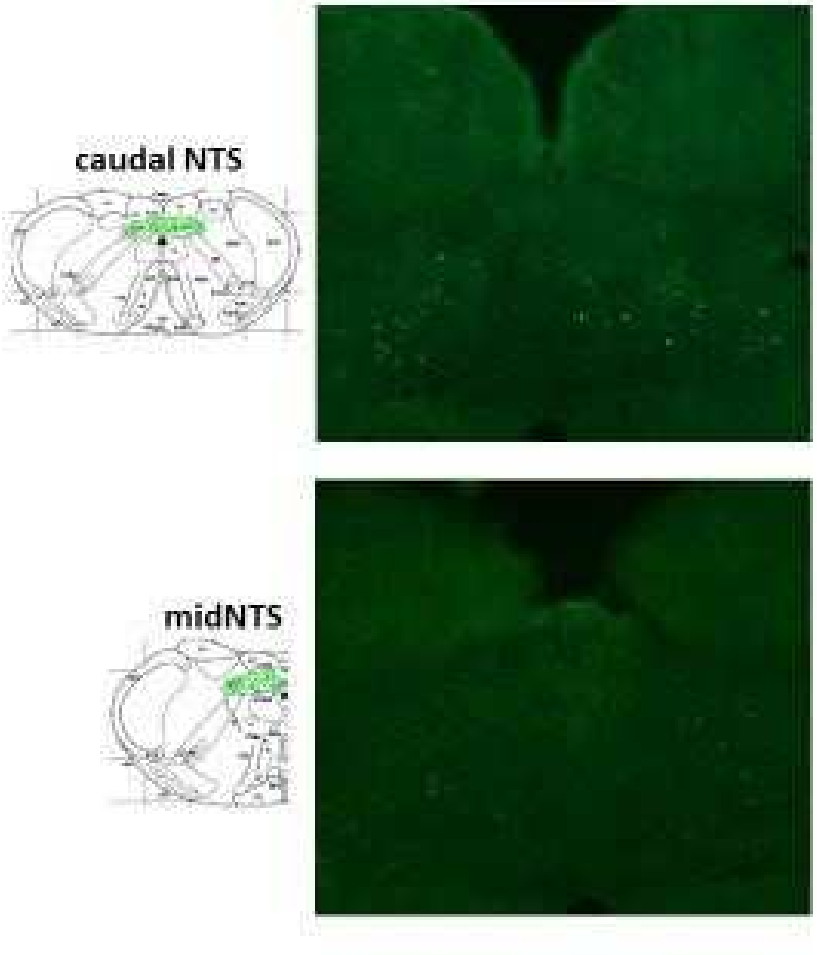


Figure 8c. Representative digital photomicrographs of ERα immunohistochemistry in the caudal (top) and mid (bottom) NTS.

Figures 9a and 9b shows quantitification of fos immunolabeling in the NTS. In both the mid (Figure 9a) and cNTS (Figure 9b), few nuclei were labeled for fos in the ad lib groups, which ate little or nothing (0-0.14 g) in the 1-hour time period prior to euthanization, or in the TTFR groups that were fasted prior to euthanization (fast). In contrast, fos immunolabeling was elevated in the NTS of the TTFR rats that were allowed to eat for 1 hour prior to euthanization (fed). Two-way ANOVAs revealed significant main effects of feeding condition in both the midNTS ($F_{(2,24)} = 17.80$, $p<0.001$) and cNTS ($F_{(2,24)} = 6.93$, $p<0.01$). Pairwise comparisons showed that, regardless of hormone treatment, numbers of fos-positive nuclei in both midNTS and cNTS were significantly greater in the fed groups than in the ad lib and fasted groups ($p<0.001$), which were not different from each other. There was no effect of hormone on numbers of fos-positive nuclei in either the midNTS or cNTS, and no interactions between hormone and feeding condition. Representative digital photomicrographs are shown in Figure 9c.

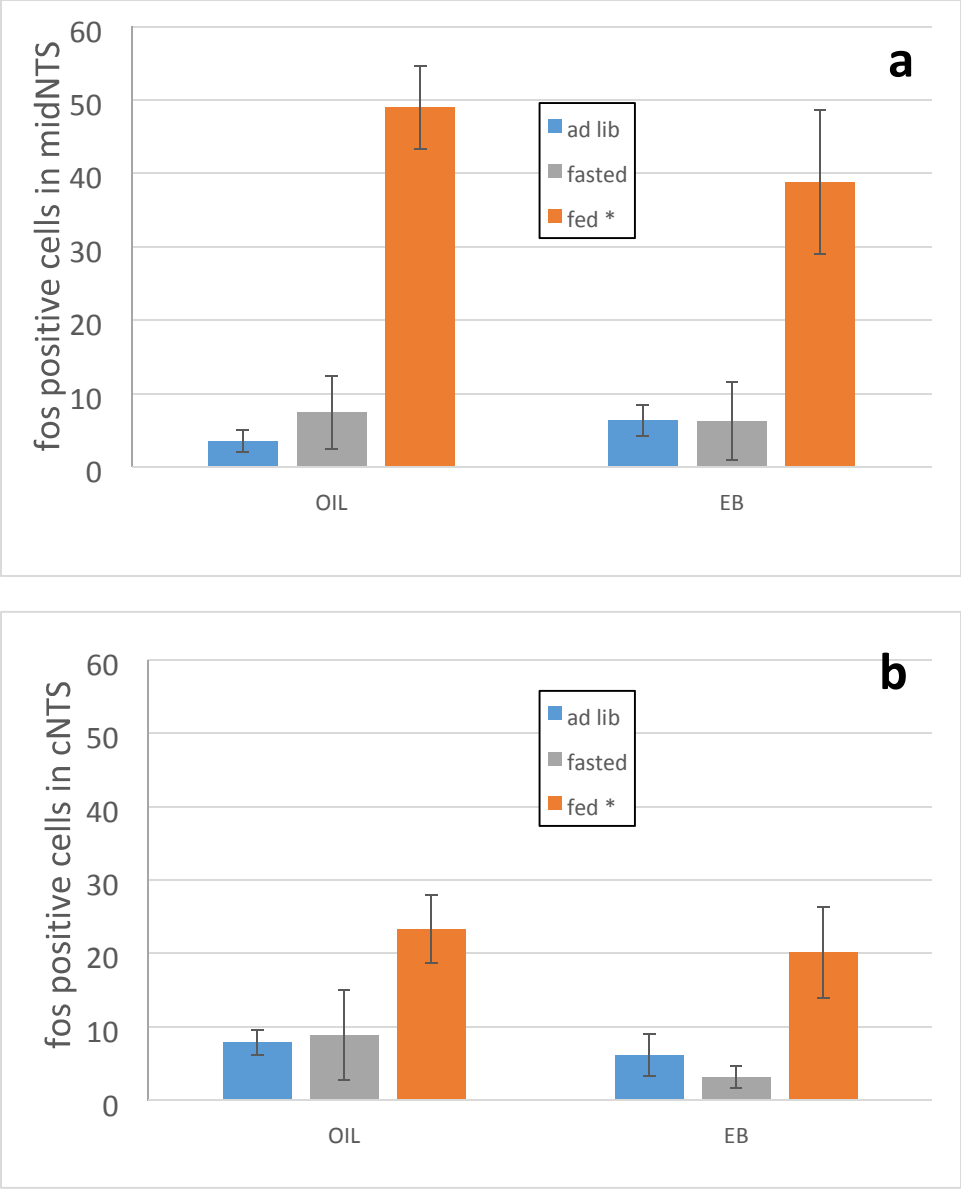


Figure 9: cFOS quantification in the NTS. 9a shows midNTS (OIL: ad lib n=5, fasted n=3, fed n=7; EB: ad lib n=4, fasted n=4, fed n=8) and 9b shows cNTS (OIL: ad lib n=5, fasted n=3, fed n=7; EB: ad lib n=4, fasted n=4, fed n=7). * = significantly greater than either the ad lib or fasted groups which were not different from each other.

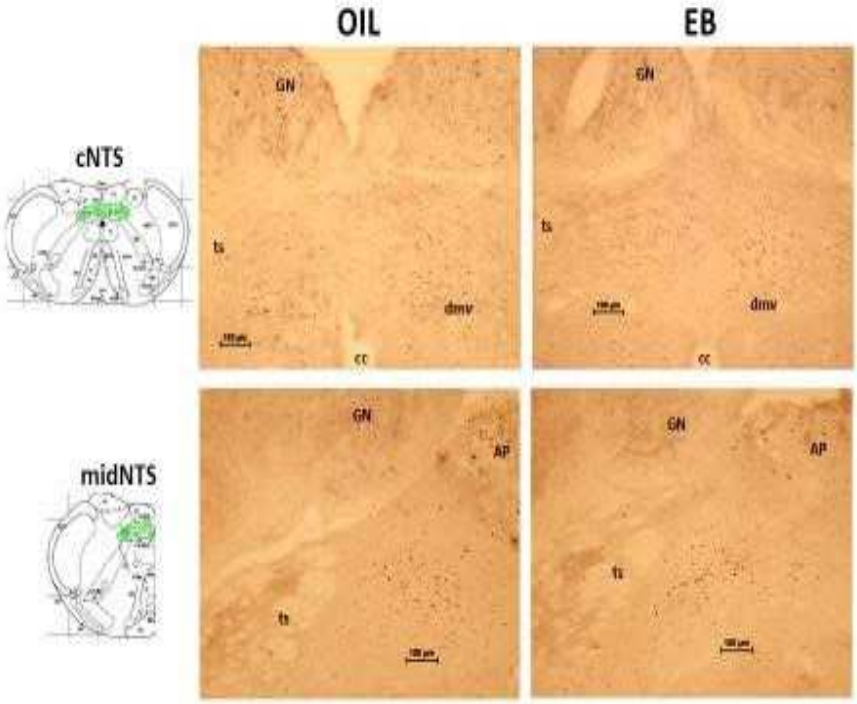


Figure 9c. Representative digital photomicrographs of c-Fos immunohistochemical labeling in the caudal (top) and mid (bottom) NTS.

Plasma insulin levels are shown in Figure 10. Two-way ANOVA demonstrated a main effect of feeding condition ($F_{(2,28)} = 11.64$, $p<0.001$), with pairwise comparisons showing that levels of insulin in the fasted group were significantly less compared to both ad lib and fed groups (ps<0.001). No hormone effect nor interaction between hormone and feeding group was seen.

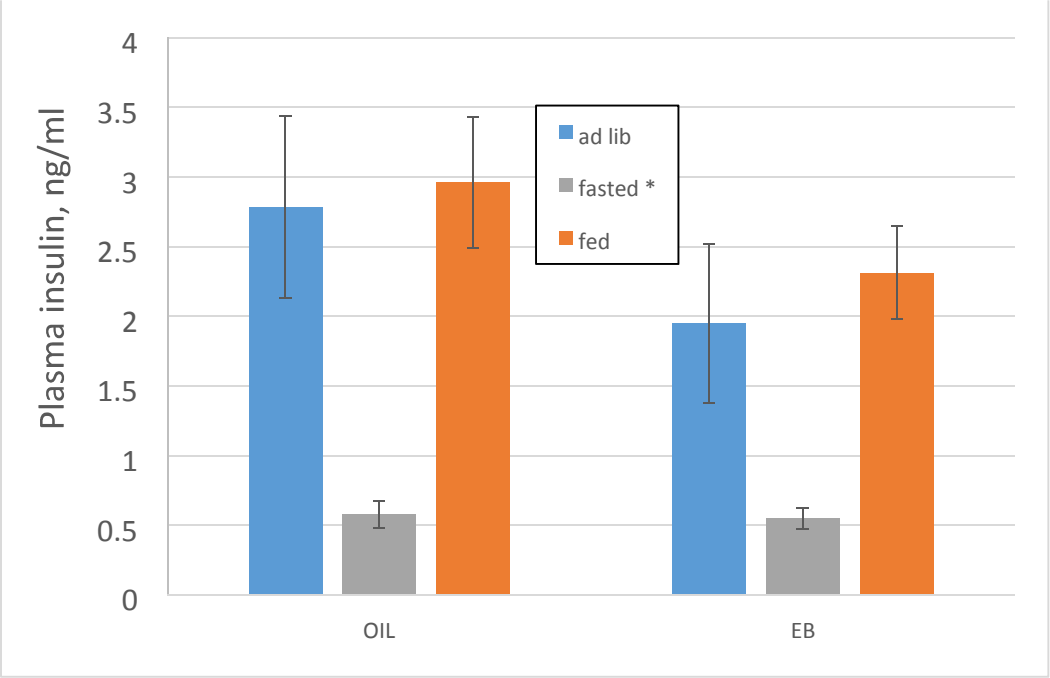


Figure 10: Plasma insulin measured on day 4 of week 3. * = significantly less than the ad lib or fed groups. OIL: ad lib n=6, fasted n=6, fed n=6; EB: ad lib n=6, fasted n=5, fed n=6.

Plasma glucose levels are shown in Figure 11. Two-way ANOVA revealed a main effect of hormone on plasma glucose concentration ($F_{(1,30)} = 14.22$, $p<0.001$,) with glucose levels in EBtreated rats significantly less than those in OIL-treated rats, independent of feeding condition. There was no effect of feeding condition, nor was there an interaction between hormone and feeding condition.

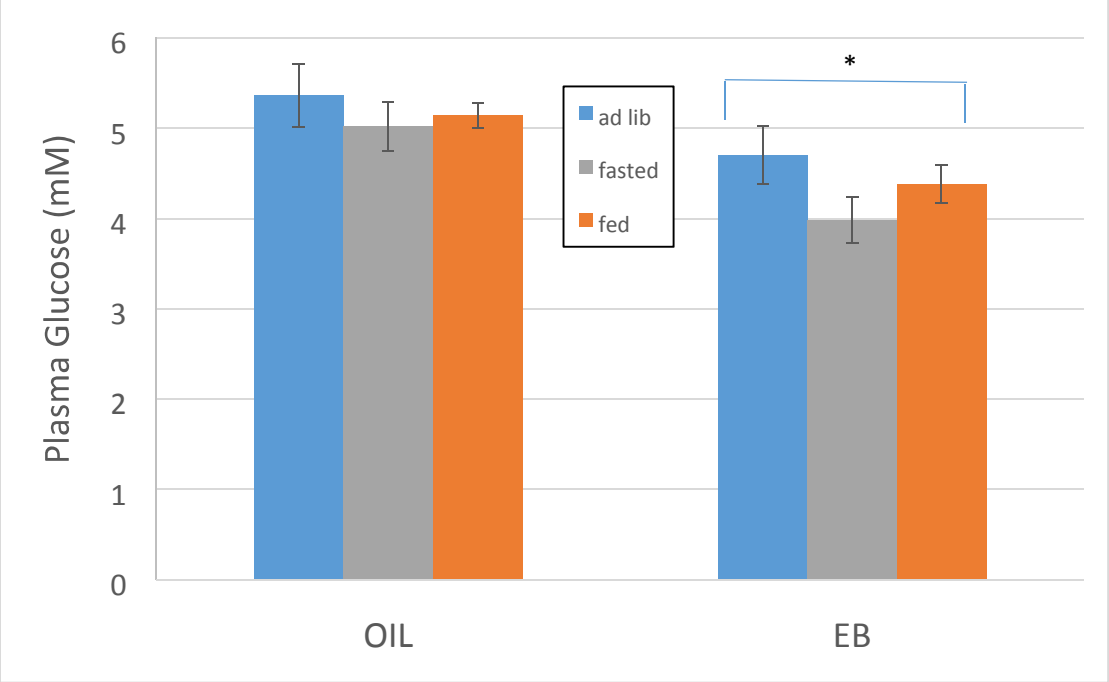


Figure 11: Plasma glucose measured on day 4 of week 3. * = significantly less than OIL-treated rats. OIL: ad lib n=6, fasted n=6, fed n=6; EB: ad lib n=6, fasted n=6, fed n=6.

Liver glycogen levels are shown in Figure 12. Two-way ANOVA revealed a main effect of feeding condition ($F_{(2,18)} = 56.03$, $p<0.001$) and pairwise comparisons showed that, independent of hormone, liver glycogen was significantly greater in the ad lib compared to that in both fed and fasted groups ($p<0.001$). In addition liver glycogen was greater in the fed group than in the fasted group ($p<0.01$). No hormone effect or interaction was shown.

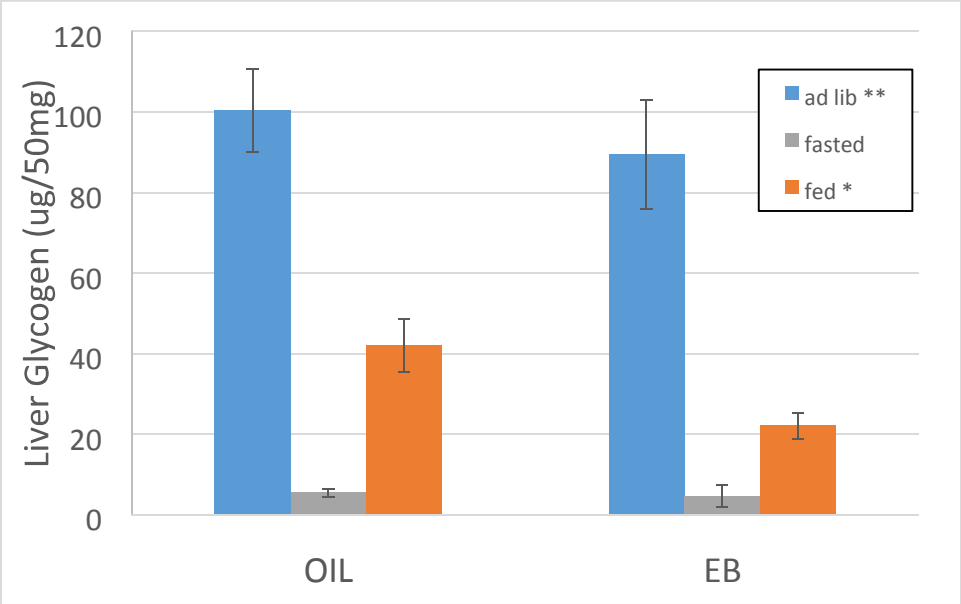


Figure 12: Liver glycogen measured on day 4 of week 3. ****significantly greater than fasted and fed groups; * = significantly greater than fasted group. OIL: ad lib n=4, fasted n=2, fed n=5; EB: ad lib n=4, fasted n=3, fed n=6.

Leptin values are shown in Figure 13. Two-way ANOVA revealed a main effect of feeding condition ($F_{(2,28)} = 7.92$, $p<0.01$) with pairwise comparisons showing that ad lib group had significantly greater leptin levels than did fasted and fed groups ($ps<0.001$). In addition, leptin levels in the fed group were significantly greater than those in the fasted group ($p<0.05$). There was no hormone effect or interaction.

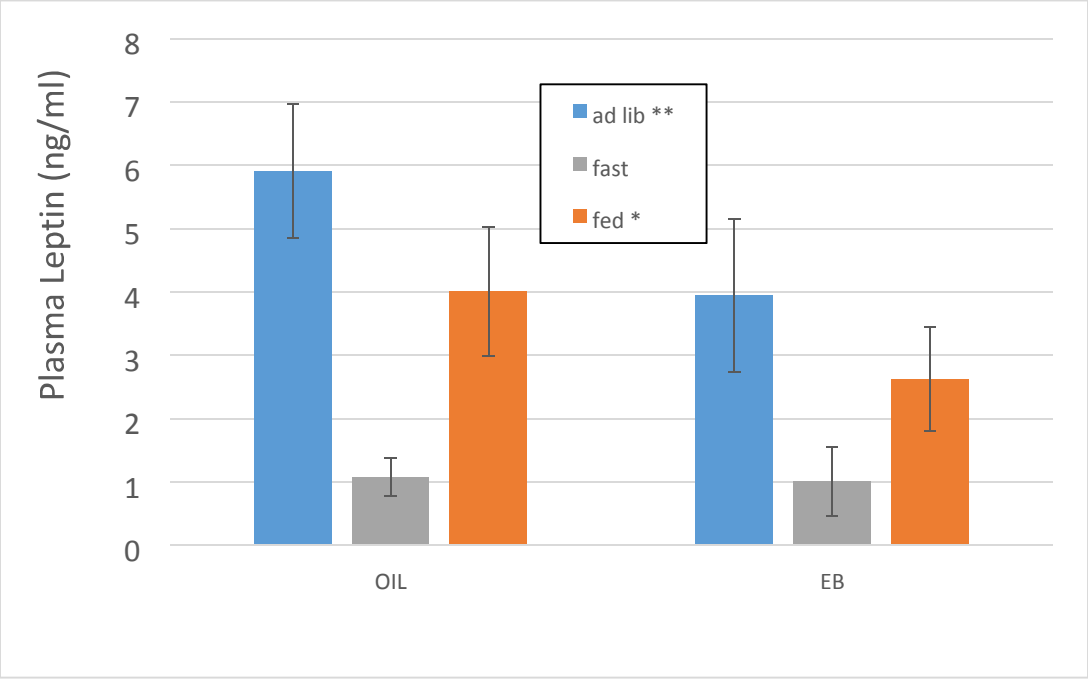


Figure 13: Plasma leptin measured on day 4 of week 3. ** = significantly greater than fasted and fed groups; * = significantly greater than fasted group. OIL: ad lib n=6, fasted n=5, fed n=6; EB: ad lib n=6, fasted n=6, fed n=6.

Plasma triglycerides are shown in Figure 14. Two-way ANOVA revealed a main effect of hormone ($F_{(1,29)}$ = 18.89, p<0.001) with plasma triglycerides in EB-treated rats significantly greater than those in OIL-treated rats. There also was a main effect of feeding condition ($F_{(2,29)}$ = 16.11, p<0.001) and pairwise comparisons showed that plasma triglycerides in the fasted group were significantly less than those in ad lib and fed groups (ps<0.001) which were not different from each other. There was no interaction between hormone and feeding condition.

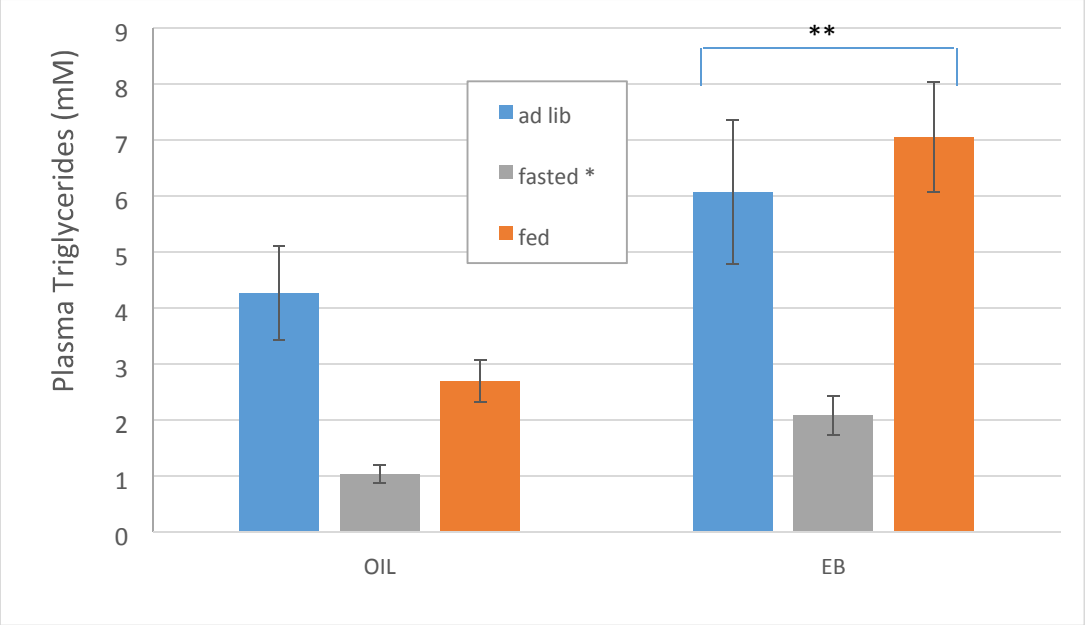


Figure 14: Plasma triglycerides measured on day 4 of week 3. ** =significantly greater than OIL-treated rats; * = significantly less than the ad lib and the fed group. OIL: ad lib n=6, fasted n=6, fed n=6; EB: ad lib n=5, fasted n=6, fed n=6.

The quantification of plasma FFA is shown in Figure 15a. Two-way ANOVA revealed a main effect of hormone ($F_{(1,30)}$ = 10.15, $p<0.01$) with plasma FFA in EB-treated rats significantly greater than those in OIL-treated. There also was a main effect of feeding condition ($F_{(2,30)}$ = 9.35, $p<0.001$), and pairwise comparisons showed that the fasting group was greater than the fed group ($p<0.001$) and greater than the ad lib group ($p<0.05$).

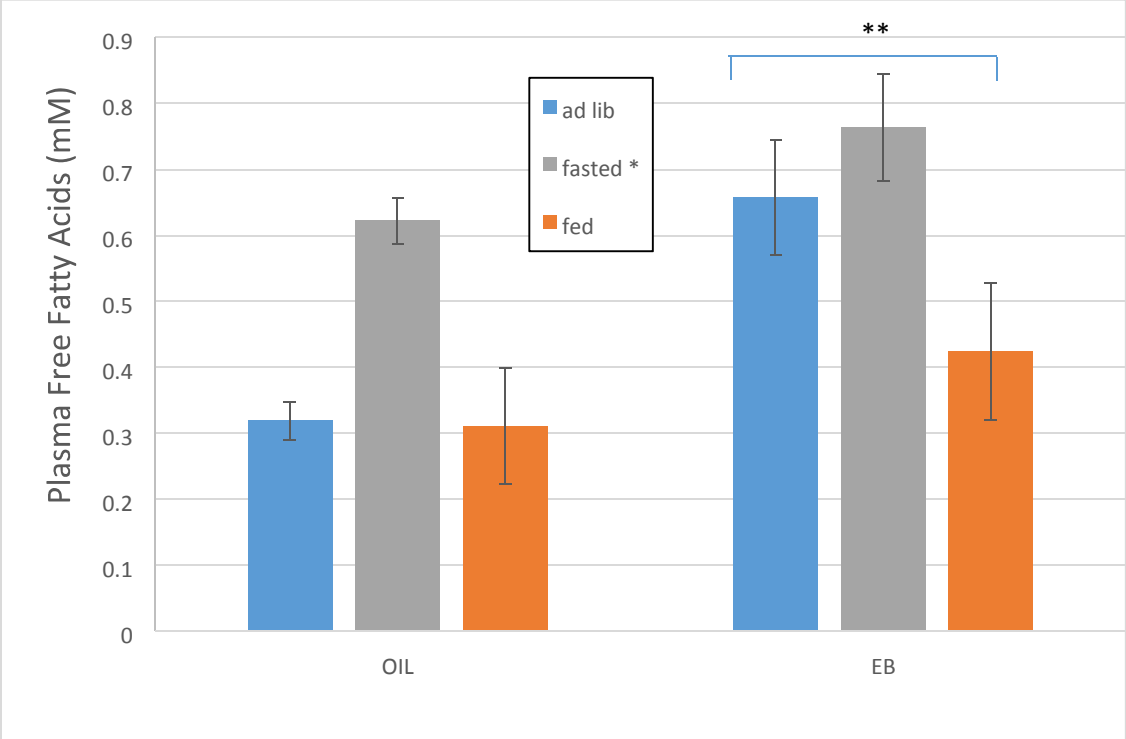


Figure 15a: Plasma free fatty acids (FFA) measured on day 4 of week 3. ** = significantly greater than OIL-treated controls. * = significantly greater than ad lib and fed groups. OIL: ad lib n=6, fasted n=6, fed n=6; EB: ad lib n=6, fasted n=6, fed n=6

Percent change in FFA is shown in Figure 15b. Two-way ANOVA revealed a main effect of feeding condition ($F_{(1,20)}$ = 17.39, p<0.001) and pairwise comparisons of feeding condition showed greater percent change in fasted animals. A main effect of hormone was also seen ($F_{(1,20)}$ = 9.85, p<0.01), with a greater percent change in OIL-treated animals. No interaction between feeding condition and hormone was seen.

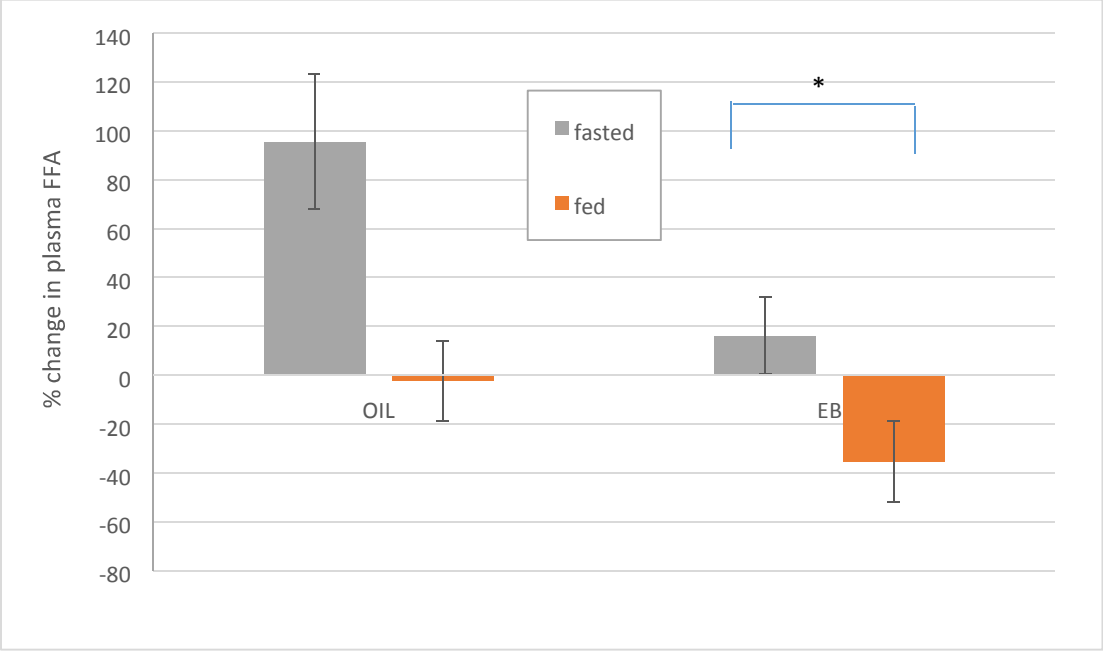


Figure 15b: Percent change from ad lib in plasma FFA measured on day 4 of week 3. * = significantly less than OIL-treated controls. OIL: fasted n=6, fed n=6; EB: fasted n=6, fed n=6.

Adipose triglyceride levels are shown in Figure 16. Two-way ANOVA revealed no main effects or interactions.

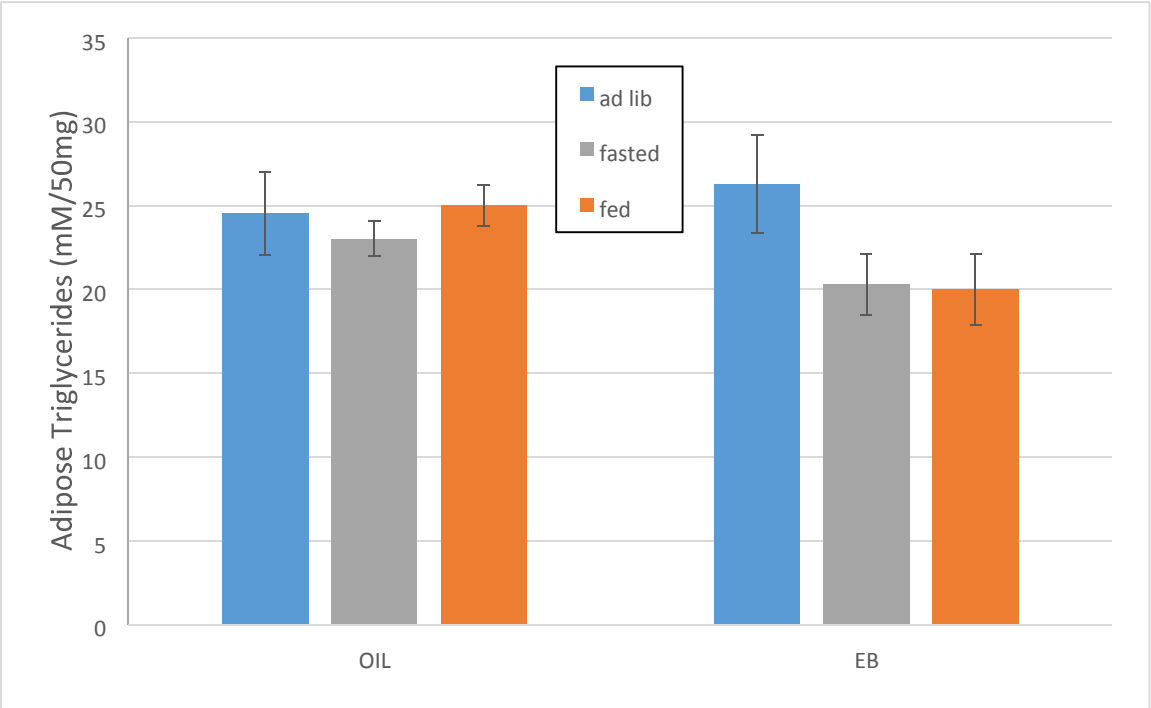


Figure 16: Adipose triglycerides measured on day 4 of week 3. OIL: ad lib n=4, fasted n=6, fed n=4, EB: ad lib n=4, fasted n=5, fed n=4.

Adrenal weights are shown in Figure 17. Two-way ANOVA revealed a main effect of hormone ($F_{(1,27)} = 18.09$, $p<0.001$), with EB-treated animals having greater adrenal weights than did OIL-treated controls. There were also was a main effect of feeding condition ($F_{(2,27)} = 13.01$, $p<0.001$) and pairwise comparisons revealed that adrenal weights in the ad lib group were significantly less ($p<0.001$) than in fed and fasted groups, which were not different from each other. There was no interaction between hormone and feeding condition.

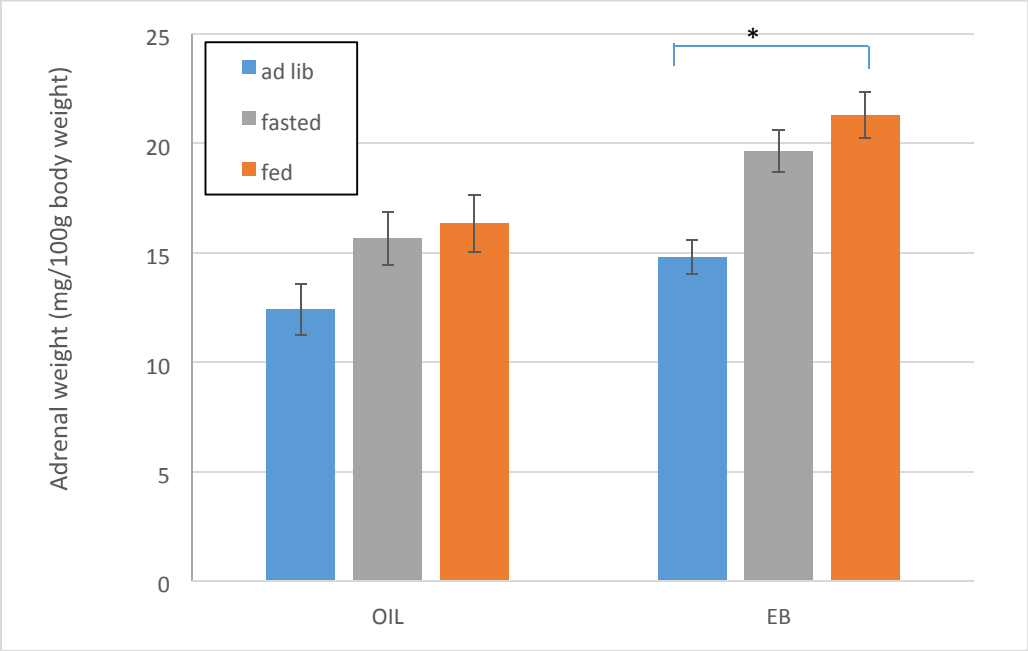


Figure 17: Adrenal weights measured on day 4 of week 3. * = significantly greater than OILtreated controls. OIL: ad lib n=6, fasted n=5, fed n=5; EB: ad lib n=6, fasted n=6, fed n=5.

Plasma corticosterone levels are shown in Figure 18. Two-way ANOVA revealed main effects of feeding condition ($F_{(2,22)} = 15.05$, $p<0.001$), hormone ($F_{(1,22)} = 56.91$, $p<0.001$) and an interaction between feeding condition and hormone ($F_{(2,22)} = 10.02$, $p<0.001$). Pairwise comparisons of the interaction revealed no differences in the corticosterone levels in the two ad lib groups, but in fasted groups and fed groups plasma corticosterone in EB-treated rats was greater than that in OIL-treated rats (ps<0.001). Plasma corticosterone of EB-treated ad lib group was less (ps>0.001) than that of fasted and fed groups, while plasma corticosterone of the fasted group was greater than that of the fed group ($p<0.05$) in the EB-treated rats. There were no differences in the OIL-treated groups between feeding conditions.

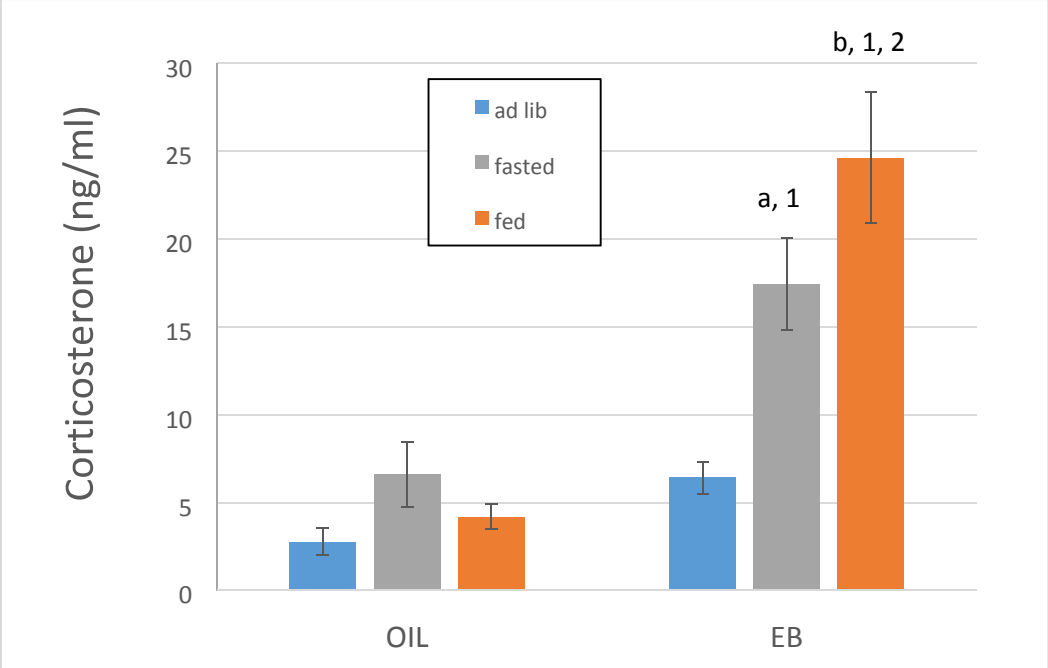


Figure 18: Plasma Corticosterone measured on day 4 of week 3. a=significantly greater than OIL fasted, b=significantly greater than OIL fed, 1=significantly greater than EB ad lib, 2=significantly greater than EB fasted. OIL: ad lib n=4, fasted n=5, fed n=5; EB: ad lib n=6, fasted n=4, fed n=4.

Table 1. Summary of Results Affected by EB treatment

Decreased by EB treatment	Increased by EB treatment
Plasma glucose (overall)	Plasma Triglycerides (overall)
	Plasma Free Fatty Acids (overall)
	TTFR Fasted plasma Free Fatty Acids
	Adrenal Weights (overall)
	TTFR Fasted plasma Corticosterone
	TTFR Fed plasma Corticosterone

CHAPTER V

DISCUSSION

Over the last 40 years, the role of estrogens in feeding and body weight regulation has become the subject of intensive investigation. From seminal observations of feeding behaviors in rats by Wade and colleagues in the mid-1970s (17, 52, 168) to more recent studies employing sophisticated techniques to explore the molecular bases of the actions of estrogens in the control of ingestive behaviors (115), work in this area has led to better understanding of the control of feeding and body weight during *ad libitum* feeding conditions. Indeed, investigators are beginning to dissect out specific CNS areas, neurotransmitter systems, and estrogen receptor subtypes (53, 73, 142, 149-151), that will allow manipulations for even more finely-honed approaches to understanding the mechanism(s) of the actions of estrogens, especially as it pertains to the CNS. Certainly, there are conflicting findings about the location of central estrogen receptors that control feeding and body weight (73, 151), and the relative influence of specific central neurotransmitter systems remains to be determined (53, 142, 148-150). Nonetheless, remarkable progress has occurred, particularly in regard to the control of feeding during conditions of *ad libitum* access.

To my knowledge, this is the first comprehensive investigation focused on the influence of estrogen on chow intake and body weight of OVX rats during TTFR. Given the propensity of overweight and obese individuals to eat only one large meal at the end of the day, when energy

levels are low (14, 46, 159), and the fact that females suffer disproportionately from obesity, the goals of this study, therefore, were to examine the effects of estradiol during TTFR and to test CNS and peripheral mechanisms that may underlie these effects. Better understanding of interactions between estradiol and inhibitory signals during TTFR has the potential to provide important insights for weight control in humans.

Estradiol Effects on Body Weight and Uterine Weights

In this study, *ad libitum* fed rats that were EB-treated weighed less than did OIL-treated rats throughout the three weeks of the study (Figure 4a). These findings of weight loss in EBtreated rats but weight gain in OIL-treated rats (Figure 3) are consistent with previous work in our lab and others (48, 79). In addition, uteri assessed three weeks after EB-treatment were hypertrophied as compared to those in OIL-treated rats (Figure 2). These measures serve as bioassays to demonstrate the efficacy of the EB-treatment.

Estradiol Effects on Chow intake and Body Weight during TTFR

During *ad libitum* access to chow, the intake that occurred overnight appeared to be satiating, as neither EB- nor OIL-treated group ate appreciable amounts of chow during 2 hour tests conducted during the day (Figure 6). In contrast, both EB-treated and OIL-treated rats consumed substantial amounts of chow in 2-hour intake tests during TTFR. Given numerous reports from our lab and others that EB decreased food intake (8, 10, 22, 48, 64, 88), it was surprising that OVX rats given EB ate the same amount of chow as did rats given OIL. These findings suggest that estradiol affects feeding during TTFR differently than it does during *ad libitum* access to chow. Thus, one would predict changes in body weight that reflect eating.

However, even though EB-treated rats weighed less than OIL-treated rats at the end of the *ad libitum* feeding period, and despite substantial body weight loss during TTFR in both groups, particularly on the first day (Figure 4b), EB-treated rats lost more weight throughout TTFR than did OIL-treated rats (Figure 5).

Mechanisms Underlying Estradiol Effects during TTFR

Estradiol effects on gastric and central mechanisms

During *ad libitum* feeding conditions, EB decreases chow intake by decreasing meal size (17, 64). This effect is thought to be due, in part, to enhanced sensitivity to gastric distension and/or the gut hormone, CCK (9, 37, 49-51, 111). In the present study, both OIL- and EB-treated rats consumed substantial amounts of chow during TTFR. In fact, amounts of chow consumed in 2-hour intake tests were approximately half the amounts typically consumed by rats during 24 hours of *ad libitum* access to chow (76), but far in excess of what would typically be consumed in a single meal during *ad libitum* feeding (8, 17). These intakes likely generated considerable gastric distension and, thereby, considerable activation of stretch receptors that send inhibitory signals to the NTS (45, 86, 119). The consumption of such large amounts of chow by OIL- and EB-treated rats in the 2-hour intake tests during TTFR suggests that both OIL- and EB-treated rats were less sensitive to inhibitory signals, particularly those related to gastric distension. Consistent with this idea, both the sensitivity (86) and the phenotype of gastric vagal afferents (45) depend on feeding condition and the amounts of food consumed. Even with such changes, however, extreme gastric stretch due to consumption of large amounts of chow such as we observed in the 2-hour intake tests would be expected to increase vagal signaling to the CNS and, ultimately, to terminate feeding (39, 86).

Gastric stretch is detected by vagal afferent fibers arising in the stomach that terminate in the NTS (33) and stimulation of those fibers produces neuronal activation in the NTS (24, 45, 50, 51, 64, 79). Therefore we opted to employ immunohistochemical labeling of the fos protein as a strategy to assess neuronal activation in the NTS in response to consumption of chow during TTFR (Figure 9a,b,c). There was little fos immunolabeling in the NTS of EB- or OIL-treated rats that had *ad libitum* access to chow and therefore ate little in the test before euthanize. Similarly, fos immunolabeling in the NTS of rats on TTFR that were not permitted to eat before euthanize (fasted) was sparse (50), an important finding which suggests that the TTFR itself did not elicit neuronal activation in the NTS. In contrast, fos immunolabeling in the NTS of rats on TTFR that were permitted to eat before euthanization (fed) was elevated, but there was no difference between hormone treatments. This finding suggests no difference in the detection of inhibitory gastric distension signaling attributable to estradiol.

Since our experimental design was intended to mimic eating patterns of some obese and overweight people who often eat only one large evening meal, we restricted the time rats were permitted to eat, rather than restricting the amount of chow provided. This approach revealed similar food intake and fos immunolabeling after EB-treatment. In contrast, Eckel and colleagues (50), reported greater fos labeling in EB-treated rats for which meal size was controlled so both EB- and OIL-treated groups ate the same amount under *ad libitum* conditions.

Estrogen effects on feeding under *ad libitum* feeding conditions are mediated in part by binding to the subtype of ER in the NTS (162). We saw no differences in the ERα immunolabeling in the midNTS or cNTS as a result of feeding condition or hormone treatment (Figure 8a,b,c). Thus, there no differential effect of either TTFR *or* EB-treatment on ERα numbers in the NTS, suggesting food intake during TTFR does not involve ERα signaling in the NTS. Though ERα and fos labeling were not done in the same tissue sections because the primary antibodies were raised in the same

animal, I found less ERα labeling in both the cNTS and midNTS than fos labeling. This suggests that there are more neurons activated (fos labeled) under the tested conditions than there were neurons containing ERα. It is possible that ERα in other CNS areas may play a role in the control of feeding with TTFR and ongoing studies will address that issue. However, it should be noted that ERα *numbers* may not reflect affinity of the receptors to bind estradiol. Thus, we cannot rule out a role for ERα in the control of feeding during TTFR, though it seems unlikely that this involves ERα in the NTS.

Together, these findings suggest that, during TTFR, EB has no effect on food intake or in the processing of signals related to the control of feeding at the level of the NTS. However, this interpretation rests on the assumption that the ingested chow is emptied from the stomach at comparable rates. In other words, even though food intake was similar between the hormone conditions during TTFR, it is possible that gastric emptying was different. Estrogen has been reported to decrease gastric emptying (25). Therefore, we weighed stomach contents on day four of TTFR as an indicator of gastric emptying and found no differences between EB- and OIL-treated rats (Figure 7). These findings suggest that there are no differences in stretch signals or processing of food through stomach between EB-treated and OIL-treated rats during TTFR.

In summary, then, our observation that numbers of fos immunolabeled neurons in the NTS of EB-treated rats were comparable to that in OIL-treated rats suggests that, unlike EB effects during *ad libitum* conditions, detection of meal-induced inhibitory signals in the NTS after TTFR is not affected by EB. However, although neural activation was not quantitatively different, different types of neurons may be activated. If this is the case the effect is unlikely to be attributable to ERα in the NTS which were not affected by EB-treatment with or without TTFR.

Estradiol effects on metabolism

Since changes in neither CNS processing nor gastric emptying are associated with EB effects during TTFR, it is possible that estrogen-mediated differences in metabolism could explain the difference in EB effects during TTFR, especially in regard to body weight loss. Accordingly, we investigated effects of EB treatment on carbohydrate and fat metabolism. In these studies we focused on primary metabolites associated with carbohydrate and fat metabolism, and on hormones related to metabolism of carbohydrates and fats.

Carbohydrate metabolism

Insulin is a major regulator of glucose levels in carbohydrate metabolism. Plasma insulin levels were not different between OIL-treated and EB-treated rats (Figure 10) but were significantly different between feeding conditions. Specifically, insulin levels in OIL-treated and EB-treated rats in the ad lib groups approximated physiological values, as also reported in other studies (56), with a trend towards lower levels in EB-treated rats. Insulin levels in both fasted groups were reduced as expected, given the decrease in glucose influx during TTFR with fasting. In contrast, insulin was restored to *ad libitum* levels within the short (one hour) time of refeeding in both hormone groups, presumably because of increased glucose influx. Thus, the hormonal regulator of glucose homeostasis, insulin, was not affected by EB-treatment during TTFR. To investigate circulating carbohydrates, we first measured plasma glucose in the OIL- and EBtreated rats. Figure 11 shows that glucose levels were significantly less in EB-treated rats, independent of feeding condition. This decrease in plasma glucose with EB treatment has been previously reported (105). However, the decreased glucose was not to the point of hypoglycemia and, in fact glucose is tightly regulated since this is a critical metabolite for brain function.

Nonetheless, the lower glucose levels in EB-treated animals may reflect an overall increase in metabolic rate, or possibly impaired gluconeogenesis leading to differences in glucose stored as glycogen.

Stored carbohydrate in the form of liver glycogen (Figure 12) was reduced in the fasted condition of both EB- and OIL-treated rats likely because it is utilized to maintain plasma glucose levels during TTFR. However, glycogen levels were partially restored within one hour after eating to about half the levels observed during *ad libitum* conditions in OIL-treated rats. The increase after eating in EB-treated rats was somewhat blunted (only to about a quarter of that during *ad libitum* conditions), though this difference was not significant. In fact, there was no effect of EBtreatment on liver glycogen. Thus, differences in carbohydrate metabolism do not appear to be an important factor determining reduced body weight of EB-treated rats during TTFR.

Lipid metabolism

The hormone leptin is released from adipocytes and is proportional to body adiposity. Despite decreased body weight in EB-treated rats during both *ad libitum* conditions and TTFR, EB did not affect circulating leptin, though there was a tendency toward reduced levels. In contrast, feeding condition exerted a profound effect on leptin (Figure 13). Plasma leptin during *ad libitum* conditions was within physiological range as previously reported (172) and leptin levels in the TTFR fasted groups were reduced, presumably because of the decreased adiposity in fasted animals. Interestingly, leptin levels in TTFR fasted groups were restored to nearly *ad libitum* levels within the short refeeding time period. This effect may be attributable to the intraluminal source of leptin (11, 27, 157). Thus, there appears to be a rapidly occurring increase in leptin which originates in the GI tract, in addition to the leptin from adipose tissue which serves as a long-term adiposity

signal. In any case neither the intra-luminal nor the adipose leptin was affected by estrogen in these studies.

A role for estrogen in fat metabolism during TTFR is suggested by previous studies of both women and rats (105). In those studies of EB-treated of OVX rats or postmenopausal women, fasted plasma triglycerides were elevated. I found that plasma triglycerides in EB-treated rats were greater in all feeding conditions compared to OIL-treated controls, an observation that may be due to the slight decrement in insulin levels with EB treatment that likely reduces lipolysis. In addition, feeding condition affected plasma triglycerides with TTFR-fasted groups having less triglycerides than did *ad libitum* and TTFR-fed groups. These data suggest that the triglycerides are being utilized for energy during fasted conditions in both EB- and OIL-treated rats. However, plasma triglycerides were restored to levels comparable to those in *ad libitum* conditions within one hour after eating during TTFR. Thus, EB-treatment increases triglycerides, but the triglyceride levels nonetheless exhibit a pattern of changes during TTFR similar to that observed in OIL-treated rats (Figure 14).

Like plasma triglycerides, plasma FFA were elevated in the EB-treated animals independent of feeding condition (Figure 15a). In addition, independent of hormone condition, plasma FFA in TTFR-fasted groups were greater than *ad libitum* and TTFR-fed groups. These observations suggest that FFA are not recruited for energy metabolism in TTFR-fasted animals. Rather, the elevated FFA in the TTFR-fasted groups may derive from the breakdown of the triglycerides, which are less in the TTFR-fasted groups. Given the difference in *ad libitum* levels of FFA between EB- and OIL-treated rats, we decided to investigate change in plasma FFA. The change calculated was less in EB-treated rats than it was in OIL-treated rats (Figure 15b), suggesting less change in plasma FFA during TTFR in EB-treated rats.

Adipose triglycerides were not affected by either hormone group or feeding condition (Figure 16). Thus, this source of energy does not seem to be utilized during TTFR. Adipose triglycerides in EB-treated TTFR groups tended to be less than those in the *ad libitum* EB-treated group. This may suggest a slower rate of uptake for the triglycerides into adipose tissue, which would be consistent with increased plasma triglycerides in EB-treated rats. Alternatively, lipolysis and mobilization of FFAs may be accelerated in EB-treated rats during TTFR.

Corticosterone/adrenals

It is known that the stress hormone, corticosterone also affects carbohydrate, fat and protein metabolism. This observation is particularly important since the TTFR schedule may serve as a physiological stressor. Thus, we weighed adrenal glands and assayed plasma corticosterone in this study. The adrenals were significantly hypertrophied in the EB-treated group compared to the OIL-treated controls and weighed more in the TTFR groups than in the *ad libitum* groups, regardless of hormone (Figure 17). Not surprisingly, given the brief feeding period, adrenal weights in TTFR-fed and TTFR-fasted groups were not different. Thus, the increase in adrenal weight in the EB-treated groups may be an indication of physiological stress during TTFR that is exaggerated by estrogen.

Plasma corticosterone levels were greater in EB TTFR-fasted and EB TTFR-fed groups than OIL TTFR-fasted and OIL TTFR-fed groups, as shown in Figure 18, possibly indicating EB treatment acts as a stressor under these conditions. Additionally, however, plasma corticosterone in EB TTFR-fasted and EB-TTFR-fed groups were greater than the EB *ad libitum* corticosterone level. The EB TTFR-fed group plasma corticosterone was greater still than that in EB TTFR-fasted group. The fed condition had the highest corticosterone level and thus, not only TTFR but also the

consumption of a large meal appears to be a physiological stressor. There was no difference in the plasma corticosterone levels in any of the OIL-treated feeding conditions. Apparently the EBtreatment per se was not responsible for the stress since the *ad libitum* EB-treated group plasma corticosterone was not different from that of the OIL-treated groups. Thus, in EB-treated rats, increased corticosterone may be responsible for differences in lipid metabolism, increased basal metabolism and thereby decreased body weights.

Summary

In summary, EB-treatment during TTFR reduces body weight without decreasing the amount of food eaten. This finding was not explained by CNS effects or gastric processing. Investigations of peripheral metabolic status of these animals, revealed no differences between EB- and OIL-treatment in the carbohydrate/insulin condition of these rats nor was leptin different between hormone groups. However, FFA and TG were elevated in EB-treated rats. These findings suggest that these EB-treated rats are utilizing lipids as did male rats on the TTFR protocol (70). In that study, the large load of nutrients taken in during TTFR were not stored as glycogen. Rather, these newly synthesized lipids, were presumably used as fuels, a shift that was proposed to be due to adapting to TTFR. This adaptation involved catabolizing fat as the major energy source and limiting glucose utilization to the small amounts required by the CNS (70). Finally, thus, elevated corticosterone in the EB-treated TTFR rats may increase the basal metabolic rate (40) resulting in greater and more efficient energy utilization, particularly of fats, and thus, decreased body weights.

Future Directions

To further these experiments, I would increase the duration of TTFR to three or four weeks to see if the weight difference between EB-treated and OIL-treated rats persists. I expect the amount eaten in a two hour period may increase to a point of stomach fullness above which it cannot go further. At that point, not only the amount eaten but also the weight loss may be the same between the groups, a fundamental change in the way estrogen effects the controls of weight loss and food intake.

I would also want to understand the underlying mechanism behind the weight differences despite similar food intake. Experiments in which dexamethasone would be used to block the corticosterone increase and thereby eliminate differences in lipid metabolism could be conducted. Additionally, if as I propose, corticosterone increases basal metabolic rate, the rates of metabolism could be measured by various techniques including assessment of VO2 max. Alternatively, an indicator of increased energy expenditure that could contribute to the increased body weight loss is to assess locomotor activity by video-taping the rats during the TTFR period.

All of these measures would serve to add to the conclusions of these experiments and lead to other questions. As always, there are more questions to be asked than there are people and time to answer them.

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VITA

Rebecca Jane Naukam

Candidate for the Degree of

Doctor of Philosophy

Thesis: EFFECT OF ESTROGEN ON TEMPORALLY TARGETED FOOD RESTRICTION: ROLE OF GASTRIC, HORMONAL AND METABOLIC FACTORS

Major Field: Biomedical Sciences

Biographical:

Education:

Completed the requirements for the Doctor of Philosophy/Education in Biomedical Sciences at Oklahoma State University Center for Health Sciences, Tulsa, Oklahoma in May, 2015.

Completed the requirements for the Master of Theological Studies at Phillips Theological Seminary, Tulsa, Oklahoma in 2010.

Completed the requirements for the Bachelor of Science in Physiological Sciences at Oklahoma State University, Stillwater, Oklahoma in 1976.

Experience:

Adjunct Instructor, Physiology,Tulsa Community College from 2010 to present.

Substitute Teacher, Certified in Secondary Science, 2009 to present

Professional Memberships:

Society for Neuroscience, student membership
Experimental Biology, student membership
American Physiological Society, student membership
Oklahoma Physiological Society, student membership
Organization for the Study of Sex Differences, student membership
Society for the Study of Ingestive Behavior, student membership
Biomedical Sciences Graduate Student Association, treasurer