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The Roles of Ovarian Steroids, Mesolimbic Dopamine, and Gonadotropin-inhibiting Hormone in Regulating Sexual and Ingestive Motivation.

by

Candice M. Klingerman

A Dissertation

Presented to the Graduate and Research Committee

of Lehigh University

in Candidacy of the Degree of

Doctor of Philosophy

in

Integrative Biology

Department of Biological Sciences

Lehigh University

January 2012

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Candice M. Klingerman

Approved and recommended for acceptance as a dissertation in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Candice M. Klingerman

"The Roles of Ovarian Steroids, Mesolimbic Dopamine, and Gonadotropin-inhibiting Hormone in Regulating Sexual and Ingestive Motivation."

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Dedication

This dissertation is dedicated to my friends and family.

I would like to thank my mother, father, grandma, grammie, and grandpap for all of their financial and emotional support through my nearly 25 years of education. Next, I thank the University of Delaware Ruminant Nutrition crew: Limin Kung, Erin McDonell, Renato Schmidt, and Richard Morris for their invaluable friendship and for teaching me how to think and act like a scientist. I thank my friends from the Lehigh University Biology Department: Jen Golley, Kim Little, Joe Leese, Kevin Tong, Tim Garelick, Marie Maradeo, Holly Richendrfer, Jeannie Smith, Jeremy Brozek, Noah Benton, Maria Brace, Jennifer Swann, Michael Burger, and Vicki Waldron for their support, friendship, and attention when I felt the need to complain or boast. I also thank my Wednesday evening trivia team: Carrie and Steve Sweeney, Jeff Spirko, Bruce Carney, and Danielle and Rachel Ringhoff for forcing me to take a night off to relax with friends and kick butt in trivia! (I love cheese!) I also thank my Ph.D. dissertation committee members John Nyby, Murray Itzkowitz, Bob Meisel, and my wonderful advisor and academic mother, Jill Schneider for their advice and guidance through my doctoral career. Lastly, I want to thank my fiancé, Steven Kress for his nourishing meals, love, support, understanding, and for letting me complain to a nonscientist about science.

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Abstract

The effects of estradiol and other putative anorectic peptides on sex and ingestive behavior have been well-studied, and yet their role in diverting attention from food to sex has not been examined directly, possibly because these functions are masked under conditions of energy abundance typical of the laboratory. This dissertation is unique in that it examines the roles of neuropeptide systems in the context in which they evolved; females are housed in a semi-natural environment and have access to both food and males. Female Syrian hamsters were subjected to different levels of energy restriction and the effects of ovarian steroids, meso-limbic dopamine, gonadotropin-inhibiting hormone, leptin, and insulin were tested in relation to sexual and ingestive motivation (vaginal scent marking, male preference, food hoarding) and performance (lordosis, food intake). First, sexual and ingestive motivation were affected by low energy prior to changes in performance or copulatory reward (cellular activation of nucleus accumbens and formation of a conditioned place preference to mating). Furthermore, changes in motivation occurred independent of changes to putative anorectic (estradiol, leptin) or orexigenic (progesterone, insulin) chemical messengers. However, increases in food hoarding and decreases in vaginal marking were associated with changes in gonadotropin-inhibiting hormone, a neuropeptide shown to suppress gonadotropin-releasing hormone and luteinizing hormone in hamsters, sex behavior in birds and rats, and increase food intake in rats, mice, sheep, and monkeys. Unlike other animal models, Syrian hamsters do not increase food intake after a period of reduced energy availability.

Instead, they increase their food hoarding. Thus, gonadotropin-inhibiting hormone represents an ideal candidate neuropeptide system for setting behavioral priorities in environments where energy availability varies or is unpredictable.

Chapter 1

Introduction

There is a long history of research on energy intake, storage, and expenditure, and the vast majority of this research has examined individual animals housed singly without the opportunity to engage in other behaviors, particularly reproductive behaviors. Similarly, research on sex behavior typically examines animals with no opportunity to engage in ingestive behavior. In nature, and under the conditions similar to those in which most species evolved, individuals must make a choice between eating and sex. While eating and sex can occur simultaneously, they usually do not. An evolutionary perspective on ingestive and sex behavior incorporates the notion that behaviors that have been under natural selection increase survival and reproductive success. Thus, a more accurate picture of neuroendocrine control of ingestive behavior incorporates the idea that mechanisms that inhibit eating allow animals to engage in behaviors related to reproduction, such as searching for mates, scent marking, territorial aggression, courtship, mating, and caring for offspring. Similarly, mechanisms that increase ingestive behaviors would be most adaptive if they delay reproduction when energy is scarce, thereby increasing the chances of individual survival, and stimulate reproductive processes when energy becomes available, thereby increasing long-term reproductive success.

In order to examine the underlying mechanisms that control ingestive and reproductive behavior, I used experimental designs that incorporate aspects of the natural environment of the species in question, Syrian hamsters. I examined the effects of energetic challenges on not only consummatory behaviors, such as food intake and lordosis, but also appetitive behaviors, such as food hoarding and the preference for males over food. I examined the role of peripheral hormones, including the ovarian hormones, estradiol and progesterone, the adipocyte hormone, leptin, and the pancreatic hormone, insulin. In addition, I examined the role of cellular activation in the meso-limbic dopamine system and in the gonadaotropin inhibiting hormone system in the brain. Both dopamine and gonadotropin-inhibiting hormone are neuropeptides that are known to influence both food intake and copulation, but the exact mechanism(s) of action are unknown. Dopamine and substances that act on dopamine receptors are associated with decreases in food intake and increases in sexual behavior. Gonadotropin-inhibiting hormone, in contrast, is associated with increased food intake and inhibition of the hypothalamic-pituitary-gonadal system. In order to better understand the roles of these hormones and neuropeptides, I examined changes in circulating levels of these hormones and in cellular activation in brain areas containing dopamine and gonadotropin-inhibiting hormone in female Syrian hamsters with various levels of energetic challenge. Hamsters were housed in a semi-natural environment and were provided with the option to engage in either sex or ingestive behaviors.

The overarching hypothesis of our laboratory is that the mechanisms that control appetitive aspects of ingestion and reproduction have evolved to maximize reproductive

success in environments where energy availability fluctuates, is unpredictable, and geographically patchy. Under this umbrella idea, I addressed the following questions in this dissertation:

1) Are the mechanisms that control appetitive aspects of sex and ingestive behavior more sensitive to energy availability than the consummatory aspects?
2) Do the levels of energy restriction that alter appetitive behaviors involve changes in circulating concentrations of estradiol or in sensitivity to estradiol?
3) Does energy restriction alter sexual motivation because of a decrease in the rewarding aspects of sex, and, if so, are these changes correlated with increases in cellular activation in the meso-limbic dopamine system?
4) Are changes in sexual and ingestive motivation associated with changes in gonadotropin-inhibiting hormone immunoreactivity or the putative anorectic and orexigenic peptides leptin and insulin?

Background

Syrian Hamsters as an Experimental Model

The consistent and well-characterized estrous cycle of the Syrian hamster (*Mesocricetus auratus*) makes it an excellent species to study the effects of food restriction on reproduction and ingestive behavior. The estrous cycle is 4 days in length, with ovulation and display of estrus behavior (lordosis) occurring on the evening of day 4. In the laboratory, lordosis occurs in response to an adult male during a time period that begins 1 hour before the onset of the dark phase of the photoperiod and continues until just before the onset of the light phase. Circulating levels of estradiol start out low and increase in the 3 days leading up to ovulation and estrous behavior. Lordosis occurs after a peak in circulating concentrations of estradiol and at a time when circulating concentrations of progesterone are increasing, and the most effective way to induce lordosis in ovariectomized hamsters is to treat with exogenous estradiol 48 hours before, and progesterone 6 hours before presentation of an adult, sexually-experienced male hamster. The brain areas where ovarian steroids act to induce lordosis (primarily the ventromedial hypothalamus) are well-characterized (Feder et al., 1974; Jones et al., 2002; Baranczuk and Greenwald, 1973; Lisk and Nachtigall, 1988; Takahashi and Lisk, 1983).

In addition, hamsters are an excellent model system because they exhibit specific appetitive and consummatory behaviors related to sex and feeding. The distinction between appetitive and consummatory behaviors can be useful when these behaviors are controlled by different mechanisms or are differentially responsive to environmental stimuli (reviewed by Ball and Balthazart, 2007). Appetitive sex behaviors occur

separated in time from copulation, reflect sexual motivation but not necessarily the ability to perform the sex act, serve to bring animals in close contact with opposite-sex conspecifics, and are assumed to induce arousal in the potential mating partner (Craig, 1917; Everitt, 1990; Johnston, 1974; Johnston, 1977; Lisk, 1983; Lorenz, 1950; Sherrington, 1906). In contrast to copulatory performance, paracopulatory behaviors of female rodents involve many different appetitive behaviors. For example, rats display hopping, darting, and ear wiggling in the presence of a male immediately before display of lordosis and mating. Hopping, darting, and ear wiggling in rats is dependent on both estradiol and progesterone (Spiteri et al., 2009). Ewes will follow rams, nudge them with their noses, and wave their tails perhaps to attract the attention of a male (Beach, 1976). Sows will mount boars and then stand to be mounted by them (Beach, 1976). Unlike rats, paracopulatory behaviors of hamsters and livestock are eliminated by ovariectomy and can be induced by giving only estradiol (Beach, 1976). An animals' motivation for sex can be measured more directly by examination of the animals' behavior that brings it in contact with males. For instance, females will cross an electric grid or press a bar to gain contact with a mate (McDonald and Meyerson, 1973; Meyerson and Lindstrom, 1973).

Appetitive sex behaviors in hamsters are easier to study than in rats. For example, rats alternate back and forth between paracopulatory and copulatory behaviors, whereas in hamsters, paracopulatory behavior occurs well <u>before</u> mating and are controlled by a different hormonal mechanism than lordosis. For example, motivational or "appetitive" aspects of sex in hamsters, such as vaginal scent marking, never occur simultaneously

with copulation. Syrian hamsters respond to male odors by making vaginal scent marks and by spending more time in closer proximity to males on day 3 of the estrous cycle, one full day before ovulation and the consummatory act of mating (Johnston, 1974; Johnston, 1975).

Appetitive aspects of ingestion include the latency to eat food, consumption of an unpalatable diet and food hoarding, which are highly sensitive to energetic challenges and can increase independent of the "consummatory" act of eating in Syrian hamsters (Buckley and Schneider, 2003; Schneider et al., 2007) and other hamster species (Bartness, 1990; Bartness and Clein, 1994).

Food hoarding is a natural appetitive ingestive behavior in Syrian hamsters. In their natural habitat, Syrian hamsters spend virtually every minute of their limited activity period outside the burrow foraging for and hoarding food (Gattermann et al., 2008). Previous work has shown that food hoarding fluctuates over the estrous cycle in hamsters and rats (Borker and Gogate, 1984; Estep et al., 1978; Fantino and Brinnel, 1986), but in previous studies, hoarding was not measured throughout the light:dark cycle nor was it assessed when the subjects were given a choice between food and a male mating partner.

In contrast to purely appetitive behaviors like vaginal scent marking, male preference, and food hoarding, consummatory behaviors refer to the consumption of or utilization of resources attained through appetitive behaviors (Beach, 1976). Lordosis and food intake might, in some cases, reflect both motivation and performance, but at least some aspects of hamster sex and ingestive behavior can be considered solely appetitive, and thus, are measures of motivation independent of ability or performance.

Another unique feature of Syrian hamsters is that unlike other laboratory rodents and mammals, hamsters do not exhibit post-fast hyperphagia, that is, they do not increase the amount of food eaten per unit time after they have been food-deprived and then returned to ad libitum food intake (Silverman and Zucker, 1976). Instead, hamsters have unique cheek pouches that they use to gather food as an external hoard. When food becomes available after a period of fasting, hamsters increase their food hoarding behavior far more than they increase their food intake (Bartness, 2003; Bartness, 1997; Bartness and Clein, 1994; Buckley and Schneider, 2003; Waddell, 1951). So unlike other rodents, hoarding behavior in hamsters allows for quantification of appetitive aspects of ingestion that are not confounded by the post-ingestive cues that result from eating.

Hamster Estrous Cycles and Ovarian Steroids

Estradiol and progesterone have long been known to be important in control of both sex and ingestive behavior. Estradiol, for example, facilitates ovulation, sex behavior and sexual motivation (McDonald and Meyerson, 1973; Meyerson and Lindstrom, 1973; Spiteri and Agmo, 2009; Wallen, 2001), but also decreases meal size, body weight and body fat content in laboratory rodents (Geary, 2001). Female mice that lack receptors for estradiol are hyperphagic, obese, and display deficits in sex behavior (Heine et al., 2000; Rissman et al., 1997). During the estrous cycle, natural increases in circulating estradiol concentrations are correlated with maximum fertility, mating behavior (Blaustein and Erskine, 2002; Ciaccio et al., 1979; Lisk and Nachtigall, 1988; Steel, 1981) and decreases in food intake and body weight in rats and other species

(Blaustein and Wade, 1976; Butera, 2009; Fessler, 2003; Wade and Gray, 1979; Wade et al., 1985). Food intake and body weight increase with ovariectomy and decrease with estradiol treatment in many species including women (Blaustein and Wade, 1976; Butera, 2009; Fessler, 2003; Wade and Gray, 1979; Wade et al., 1985) and the subjects of the present study, Syrian hamsters (Morin and Fleming, 1978; Schneider et al., 1986). There is a great deal of circumstantial evidence suggesting that ovarian hormones orchestrate reproduction and energy balance, but very few studies have addressed directly the functional significance of the pleiotropic effects of estradiol and progesterone.

We hypothesize that one important function of the so-called 'satiety' hormones, such as estradiol, is to set behavioral priorities for the purpose of optimizing reproductive success in natural habitats where food availability varies unpredictably (Schneider, 2006; Schneider, 2007; Schneider and Watts, 2009). According to this hypothesis, the effects of estradiol on sexual motivation are expected to differ under different levels of energy availability. Under extreme shortages, when energy demands far outstrip energy availability, reproduction is expected to be inhibited in favor of intense interest in foraging, hoarding and eating (Morin, 1986; Schneider and Wade, 1989; Schneider and Wade, 1990). Under less extreme challenges, when energy demand is high but has not exceeded availability, or when food availability is unpredictable, reproductive hormones are expected to increase sexual motivation during the fertile period and distract animals from their hunger for food. Thus, even when females must hoard food in anticipation of future shortages, the temporary increase in ovarian steroids boosts sexual motivation in order to make sex a priority over food during the peak in fertility. In contrast, when

energy availability is in excess of energy demand, the role of fluctuating ovarian hormones in overcoming hunger might be masked because hunger motivation is low relative to sexual motivation under these conditions. This idea has not been tested directly, but is supported by the following ideas from ecological neuroendocrinology. Food supplies in nature are rarely as predictable as in the laboratory and our western industrialized societies. Animals in the wild must expend considerable energy and time obtaining food, and in many cases, must anticipate future energy needs that are greater than the requirements for immediate survival (Bronson, 1989). Energy can be stored internally as adipose tissue or externally as food caches or hoards in anticipation of the increased energetic demands of pregnancy, lactation, seasonal changes in food availability or unpredictable natural disasters. Taking time and energy for reproductive activity in such energetically labile habitats can be risky because lack of vigilance to energetic demands could preclude any future genetic contribution to the next generation (Wallen, 2000). Because evolutionary adaptation involves reproductive success in addition to survival, one role of ovarian steroids is to overcome the risks involved in reproduction by making sex behavior a priority over other behaviors (Wallen, 2000). The aim of the experiments in Chapter 2 of this dissertation was to unmask the role of hormones in coordinating sex and eating by measuring several aspects of sex and ingestive behavior over the estrous cycles in Syrian hamsters housed in a setting that approximates the energetic conditions of their wild ancestors. I asked whether appetitive or consummatory behaviors are more sensitive to energetic challenges.

Metabolic Control of Reproduction and Ingestive Behavior

Metabolic control of the reproductive system has been demonstrated in every order of the class Mammalia, and some important work in this field was done in the Wade lab using Syrian hamsters as a model system. Schneider and Wade (1989) hypothesized that the function of the mechanisms that inhibit the hypothalamic-pituitary-gonadal system in the face of low availability of metabolic fuels is to set behavioral priorities that optimize reproductive success in environments where food availability and energy demands fluctuate (Bronson, 1989; Wade and Schneider, 1992). Thus, they suggested that food deprivation-induced anestrus would be mimicked by treatments that blocked metabolic fuel oxidation in Syrian hamsters (Schneider and Wade, 1989). The effects of inhibition of fuel oxidation were replicated in many different species including sheep, rats, and monkeys.

The mechanisms that switch behavioral priorities from ingestive to reproductive behaviors might occur at multiple loci. Total food deprivation inhibits the hypothalamic-pituitary-gonadal system, including the gonadotropin releasing hormone pulse generator, pituitary gonadotropin secretion and ovarian steroid secretion. Despite action at multiple loci, the majority of research has focused on metabolic factors, hormones, and neuropeptides that induce anestrus and stimulate food intake and vice versa (Cunningham, 2004; Foster et al., 1998; Henry et al., 1999; I'Anson et al., 1991; Kalra et al., 1988; McShane et al., 1992; Schneider, 2004; Wade and Schneider, 1992). Food deprivation and other metabolic challenges inhibit pulsatile gonadotropin-releasing hormone secretion that, in turn, inhibits pituitary luteinizing hormone secretion, ovarian

steroid synthesis and secretion, and ovarian-steroid-dependent copulatory behavior in a wide variety of species, including Syrian hamsters (Armstrong and Britt, 1987; Bronson, 1988; Bronson and Marsteller, 1985; Cameron, 1996; Foster and Olster, 1985; McClure, 1962; Morin, 1975; Ronnekleiv et al., 1978; Schneider and Wade, 1989; Shahab et al., 2006; Shahab et al., 1997; Sprangers and Piacsek, 1988; Temple et al., 2002; Terry et al., 2005; Thomas et al., 1990).

The effects of these severe energetic challenges on the estrous cycle have in common specific changes to estrogen receptor expression. For example, 48-hours of food deprivation or treatment with pharmalogical blockers of glycolsis (2-Deoxy-D-glucose) and fatty acid oxidation (methyl palmoxirate) in hamsters decreases estrogen receptor-α (ER-α) immunoreactivity (IR) in the ventromedial hypothalamus, a brain area necessary for display of lordosis in the hamster (Li et al., 1994). Furthermore, consistent with a decrease in food intake seen after estradiol treatment, food-deprived hamsters have higher ER-IR in the paraventricular nucleus of the hypothalamus, an area where estradiol implants decrease food intake without inducing lordosis. Similar durations of food deprivation decrease progestin receptor immunoreactivity in the medial preoptic area and amygdala, brain areas also necessary for normal sexual behavior in the hamster (Du et al., 1996).

Most investigators in the field of metabolic control of reproduction study effects of food deprivation in very lean animals to determine how metabolic deficits inhibit gonadotropin-releasing hormone and gonadotropin secretion. Very few investigators, prior to my experiments, had examined the effects of mild energetic challenges on

behavior. Schneider et al., showed that the choice between food and sex was altered in fat, food-deprived Syrian hamsters and this effect was reversed by leptin (Schneider et al., 2007). In these experiments, ad libitum-fed females showed more vaginal scent marks on day 3 of the estrous cycle than food-deprived females, even though neither group decreased their lordosis frequency or duration. Leptin treatment not only reversed the effects of food deprivation, but increased levels of vaginal scent marking above and beyond that of ad libitum-fed females. This suggests that the hormones normally thought to control satiety, such as estradiol and leptin, might actually function to orchestrate sexual and ingestive behaviors (Schneider et al., 2006; Schneider et al., 2007).

In contrast to severe metabolic challenges, my dissertation experiments examine metabolic effects on behavioral motivation (the internal desire for food or sex), performance (mating and eating) and reward (the positive affect that increases the likelihood of recurrence of the behavior). Prior to the experiments presented in this dissertation, it was not known whether the effects of mild food restriction on appetitive aspects of ingestive behavior occur via changes in circulating levels of ovarian steroids or sensitivity to ovarian steroids or other differences. Females were food-restricted by 25% in my experiments, a level of restriction that has no immediate adverse effects on health, body condition, and estrous cyclicity when compared to 50% restriction or complete food deprivation. Chapters 2, 3, and 4 address these questions.

Reward and Conditioned Place Preference

Another aspect of behavior that has not been examined in the context of variability in energy availability is reward. Reward is thought to occur whenever an experience changes the brain and behavior to make that experience more likely to happen again in the future. One method of determining reward is using the conditioned place preference paradigm. In short, an animal receives a given stimulus in a previously un-preferred compartment of an apparatus. If the experience is rewarding, the animal will continue to spend time in that compartment even when the stimulus is removed. Again, a great deal is known about the neuroendocrine processes involved in the formation of a conditioned place preference by sexual reward in Syrian hamsters. Formation of a conditioned place preference to sexual stimuli in hamsters requires the act of copulation and the presence of estradiol and progesterone (Meisel and Mullins, 2006). Copulatory performance and reward can be dissociated. For instance, a dopamine receptor (D2) antagonist blocks formation of a conditioned place preference to copulation without affecting lordosis (Meisel et al., 1996).

The meso-limbic dopamine system, including the nucleus accumbens, is thought to play a role in appetitive behavior, (Alcaro et al., 2007; Bassareo and Di Chiara, 1999; Ikemoto and Panksepp, 1999; Mitchell and Gratton, 1994; Salamone, 1994), effortful approach to reinforcing stimuli (Salamone et al., 2009), and is activated by experience with addictive drugs (Di Chiara and Imperato, 1988; Nestler, 2001) and naturally rewarding stimuli including copulatory experience (Kalivas and Volkow, 2005; Kohlert and Meisel, 1999; Lajtha and Sershen, 2010; Meisel and Joppa, 1994; Salamone et al., 2005). Whether there is increased release of dopamine and neural activation after vaginal

scent marking or other appetitive behaviors in hamsters is unknown, and since mild food restriction decreases these behaviors, I realized it would be difficult to determine whether food restriction also inhibits the rewarding aspects of these behaviors. However, there are instances where the rewarding aspects of copulation are inhibited even when the performance of copulation is not inhibited. In Syrian hamsters, for example, copulatory experience increases neural activation and dopamine release in the nucleus accumbens (Bradley and Meisel, 2001; Kohlert and Meisel, 1999), and antagonists to dopamine receptors (D2) block the rewarding aspects of sexual experience without decreasing copulatory behavior (lordosis) (Meisel et al., 1996). Perhaps food restriction, like a dopamine receptor antagonist, also decreases the rewarding consequences of copulation. This idea is examined in Chapter 3.

Hormones and Neuropeptides involved in Appetitive Sex and Ingestive Behaviors

There is a vast number of neuropeptides thought to be involved in control of ingestive behavior. Most of those that increase food intake also inhibit reproductive processes and vice versa (reviewed by Schneider et al., 2011). In my dissertation experiments I focused on estradiol and progesterone (Chapter 2), the meso-limbic dopamine system (Chapter 3), gonadotropin-inhibiting hormone (Chapter 4), and neuropeptide Y (Chapters 4). In addition, peripheral hormones leptin and insulin were examined in response to various levels of food restriction (Chapter 4).

Gonadotropin-inhibiting hormone is positioned neuroanatomically to modulate sexual and ingestive behavior. Gonadotropin-inhibiting hormone is an RFamide

produced and secreted mainly from the dorsomedial nucleus of the hypothalamus in mammals and the paraventricular nucleus of the hypothalamus in birds (Bentley et al., 2006; Kriegsfeld et al., 2006; Ukena and Tsutsui, 2001; Ukena et al., 2003). A large portion of gonadotropin-inhibiting hormone cells project from the dorsomedial nucleus/paraventricular nucleus onto gonadotropin-releasing hormone neurons within the preoptic area where the peptide suppresses gonadotropin-releasing hormone activity (Kriegsfeld, 2006). Gonadotropin-releasing hormone has well-known positive influences on reproductive behavior. Food deprivation and other metabolic challenges suppress pulsatile gonadotropin-releasing hormone which causes a cascade of inhibitory events including inhibition of pulsatile luteinizing hormone secretion, ovarian steroid synthesis and secretion, and ovarian-steroid-dependent mating behaviors (Bronson and Marsteller, 1985; Morin, 1975; Ronnekleiv et al., 1978; Schneider and Wade, 1989; Shahab et al., 2006; Shahab et al., 1997; Temple et al., 2002). Other key gonadotropin-inhibiting hormone projection sites that could influence sexual and ingestive behavior include the arcuate and paraventricular nuclei of the hypothalamus, amygdala, and median eminence (Kriegsfeld, 2006). Projections of gonadotropin-inhibiting hormone cells to the median eminence make the peptide capable of directly inhibiting luteinizing hormone secretion.

Like other putative orexigenic peptides, gonadotropin-inhibiting hormone increases ingestive behavior and also decreases sexual behavior. Injections of gonadotropin-inhibiting hormone increase food intake in a number of species including rats, sheep, mice, and monkeys (I.J. Clarke personal communication; Johnson et al., 2007) and treatment with a gonadotropin-inhibiting hormone antagonist prevents post-

fast hyperphasia in chickens (Tachibana et al., 2005). Alternatively, gonadotropin-inhibiting hormone decreases copulatory solicitation in female birds and male rats (Bentley et al., 2006; Johnson et al., 2007). In addition to suppressing gonadotropin-releasing hormone neurons, injections of gonadotropin-inhibiting hormone also prevent the luteinizing hormone surge and patterns of gonadotropin-inhibiting hormone expression vary over the estrous cycle, but are lowest around the time of the luteinizing hormone surge (Gibson et al., 2008; Kriegsfeld et al., 2006).

Estradiol and progesterone were measured because of their well-known involvement with sex and ingestive behavior. The putative anorectic hormone leptin and orexigenic hormone insulin were also measured because of their influence on behavior. Leptin and insulin decreases rapidly in hamsters prior to changes in body fat by 12 hours of fasting (Schneider et al., 2000). In theory, when an animal is well-fed, levels of leptin are high and food intake is suppressed (Schwartz et al., 2000). Animals in negative energy balance have less body fat and hence less circulating leptin and food intake is elevated (Schwartz et al., 2000). Leptin given to food-deprived, female hamsters actually increases their sexual motivation (vaginal marking), reverses fasting-induced anestrus, and attenuates food hoarding (Buckley and Schneider, 2003; Schneider et al., 2007; Schneider et al., 1998). Alternatively, peripheral injections of insulin increase ingestive and decrease sex behavior of Syrian hamsters (Rowland, 1978; Wade et al., 1991). In fact, treatment with supraphysiological insulin is one of only a few methods of inducing hyperphasia in Syrian hamsters and if female hamsters are prevented from overeating, they become anestrus (Wade et al., 1991).

Brain areas of interest must include the arcuate nucleus and paraventricular nucleus of the hypothalamus, which contain leptin receptors, neuropeptide Y/agouti-related protein, proopiomelanocortin and corticotropin releasing hormone (Backholer et al., 2009; Baldo et al., 2003; Bentley et al., 2006; Hakansson et al., 1998; Jones et al., 2004; Mercer et al., 1998; Qi et al., 2009; Seymour et al., 2005; Tartaglia et al., 1995) Neuropeptide Y gene expression is increased, particularly in the arcuate nucleus, in response to food deprivation and systemic or hypothalamic injection of neuropeptide Y cause potent increases in food intake (Clark et al., 1984; Levine and Morley, 1984; Schwartz et al., 1993; Stanley and Leibowitz, 1984) and decreased lordosis in hamsters (Jones et al., 2004). Neuropeptide Y also interacts with estradiol to affect food intake. For example, systemic injections of estradiol decrease the orexigenic effect of neuropeptide Y in rats (Santollo and Eckel, 2008) and hypothalamic explants treated with estradiol decrease neuropeptide Y gene expression by as much as 91% compared to vehicle in mice (Olofsson et al., 2009). In cycling rats, an endogenous increase in estradiol around ovulation or an injection of estradiol to ovariectomized females corresponds to decreases in neuropeptide Y and decreased food intake (Clegg et al., 2007). Neuropeptide Y released into the paraventricular nucleus of the hypothalamus, another brain area important for feeding and sex behavior, is also increased after ovariectomy and reversed by estradiol in rats (Bonavera et al., 1994).

Hypotheses

Given the above background considerations, I tested the hypothesis that hormones and neuropeptides orchestrate appetitive sex and ingestive behaviors in order to optimize reproductive success. This overarching hypothesis lead to the following specific hypotheses:

1) Appetitive ingestive and sex behaviors are more sensitive to energy availability than consummatory behaviors
2) Decreased energy availability that affects appetitive behaviors involves changes in sensitivity to estradiol rather than changes in circulating estradiol. Furthermore, estradiol is more important than progesterone for behavioral motivation.
3) Effects of energy availability on appetitive behaviors alters the rewarding consequences of sex and this is reflected in cellular activation of the meso-limbic dopamine system.
4) Changes in appetitive behavior in response to energy availability are more closely associated with changes in cellular activation in gonadotropin-inhibiting hormone cells than to circulating concentrations of leptin, insulin or ovarian steroids.

Chapter 2

Energetic Challenges Unmask the Role of Estradiol in Orchestrating Ingestive and Sex Behaviors

Estradiol and progesterone are well known reproductive hormones that have garnered attention for their effects on energy balance. Estradiol, for example, facilitates ovulation, sex behavior and sexual motivation (McDonald and Meyerson, 1973; Meyerson and Lindstrom, 1973; Spiteri and Agmo, 2009; Wallen, 2001), but also decreases meal size, body weight and body fat content in laboratory rodents (Geary, 2001). Female mice that lack receptors for estradiol are hyperphagic, obese, and display deficits in sex behavior (Heine et al., 2000; Rissman et al., 1997). During the estrous cycle, natural increases in circulating estradiol concentrations are correlated with maximum fertility, mating behavior (Blaustein and Erskine, 2002; Ciaccio et al., 1979; Lisk and Nachtigall, 1988; Steel, 1981) and decreases in food intake and body weight in rats and other species (Blaustein and Wade, 1976; Butera, 2009; Fessler, 2003; Wade and Gray, 1979; Wade et al., 1985). Food intake and body weight increase with ovariectomy and decrease with estradiol treatment in many species including women (Blaustein and Wade, 1976; Butera, 2009; Fessler, 2003; Wade and Gray, 1979; Wade et al., 1985) and the subjects of the present study, Syrian hamsters (Morin and Fleming, 1978; Schneider et al., 1986). There is a great deal of circumstantial evidence suggesting that ovarian hormones orchestrate reproduction and energy balance, but very few studies have

addressed directly the functional significance of the pleiotropic effects of estradiol and progesterone.

We hypothesize that one evolved function of the so-called 'satiety' hormones, such as estradiol, is to set behavioral priorities for the purpose of optimizing reproductive success in natural habitats where food availability varies unpredictably (Schneider, 2006; Schneider, 2007; Schneider and Watts, 2009). According to our hypothesis, the effects of estradiol on sexual motivation are expected to differ under different levels of energy availability. Under extreme shortages, when energy demands far outstrip energy availability, reproduction is expected to be inhibited in favor of intense interest in foraging, hoarding and eating (Morin, 1986; Schneider and Wade, 1989; Schneider and Wade, 1990). Under less extreme challenges, when energy demand is high but has not exceeded availability, or when food availability is unpredictable, reproductive hormones are expected to increase sexual motivation during the fertile period and distract animals from their hunger for food. Thus, even when females must hoard food in anticipation of future shortages, the temporary increase in ovarian steroids boosts sexual motivation in order to make sex a priority over food during the peak in fertility. In contrast, when energy availability is in excess of energy demand, the role of fluctuating ovarian hormones in overcoming hunger might be masked because hunger motivation is low relative to sexual motivation under these conditions. This idea has not been tested directly, but is supported by the following ideas from ecological neuroendocrinology. Food supplies in nature are rarely as predictable as in the laboratory and our western industrialized societies. Animals in the wild must expend considerable energy and time

obtaining food, and in many cases, must anticipate future energy needs that are greater than the requirements for immediate survival (Bronson, 1989). Energy can be stored internally as adipose tissue or externally as food caches or hoards in anticipation of the increased energetic demands of pregnancy, lactation, seasonal changes in food availability or unpredictable natural disasters. Taking time and energy for reproductive activity in such energetically labile habitats can be risky because lack of vigilance to energetic demands could preclude any future genetic contribution to the next generation (Wallen, 2000). Because evolutionary adaptation involves reproductive success in addition to survival, one role of ovarian steroids is to overcome the risks involved in reproduction by making sex behavior a priority over other behaviors (Wallen, 2000). Our aim, therefore, was to unmask the role of hormones in coordinating sex and eating by measuring several aspects of sex and ingestive behavior over the estrous cycles in Syrian hamsters housed in a setting that approximates the energetic conditions of their wild ancestors.

Syrian hamsters (*Mesocricetus auratus*) were used because they show consistent four-day estrous cycles that are easily monitored and because they exhibit behaviors that allow us to assess their motivations independent of the ability to perform behaviors. For example, motivational or "appetitive" aspects of sex, such as vaginal scent marking, never occur simultaneously with copulation, and appetitive aspects of ingestion, such as food hoarding, are highly sensitive to energetic challenges and can increase independent of the "consummatory" act of eating in Syrian hamsters (Buckley and Schneider, 2003; Schneider et al., 2007) and other hamster species (Bartness, 1990; Bartness and Clein,

1994). Estrous behavior (lordosis) is routinely studied in the laboratory by injections of estradiol followed by progesterone (Feder et al., 1974; Jones et al., 2002), and circulating levels of estradiol and estradiol-induced vaginal scent marking start out low and increase in the 3 days leading up to ovulation and estrous behavior (lordosis) in the laboratory (Baranczuk and Greenwald, 1973; Lisk and Nachtigall, 1988; Takahashi and Lisk, 1983), and presumably in the wild. In their natural habitat, Syrian hamsters spend virtually every minute of their limited activity period outside the burrow foraging for and hoarding food (Gattermann et al., 2008). Previous work has shown that food hoarding fluctuates over the estrous cycle in hamsters and rats (Borker and Gogate, 1984; Estep et al., 1978; Fantino and Brinnel, 1986), but in previous studies, hoarding was not measured throughout the light:dark cycle nor was it assessed when the subjects were given a choice between food and a male mating partner.

We compared appetitive and consummatory sex and ingestive behaviors in female hamsters with high, medium, and low food availability and with and without mating partners. In Experiment 2.1, we measured these behaviors in solitary female hamsters with unlimited food or food restricted. Food hoarding and intake were measured every 6 hours over the estrous cycle: the former, but not that latter was nocturnal and occured near the light:dark transition. In Experiment 2.2, we measured the choice between hoarding and sex behavior at the onset of the dark phase of the photoperiod every night of the estrous cycle in females with ad libitum access to food and made comparisons to those with either restricted food availability or limited time for hoarding and mating. According to our hypothesis, females living in conditions of energy scarcity were

expected to show high levels of food hoarding and low interest in males on all nights of the estrous cycle except the night of proestrous when they were expected to show the opposite pattern. Our hypothesis predicted that hamsters living in energy abundance would have little variation in food hoarding over the estrous cycle and prefer to spend their time assessing the whereabouts of conspecifics every night. Conversely, our hypothesis would be refuted if food availability had no effect on estrous cycle fluctuations in hoarding and male preference. In Experiment 2.3, females were ovariectomized and treated with estrous-inducing doses of estradiol and progesterone or vehicle to confirm that the results of Experiment 2.2 were due to these hormones. Furthermore, Experiment 2.4 compared the effects of estradiol with and without progesterone in females tested first in the presence of males and then in the absence of males.

Materials and Methods

Animals and Housing

Experiments were conducted according to the American Physiological Society guiding principles for research, the National Institutes of Health, the Lehigh University Institutional Animal Care and Use Committee, and the United States Department of Agriculture. Female Syrian hamsters (*Mesocricetus auratus*) weighing between 100 g and 160 g were obtained from a colony bred at the Lehigh University animal facility or purchased from Charles River Breeding Laboratory (previous generations of animals were also obtained from Charles River Breeding Laboratories; Wilmington, MA).

Animals were singly housed in opaque, Nalgene cages (31 × 19 × 18 cm) in a room maintained at 22 ± 1°C with a 14:10 light-dark cycle (lights on at 2100 h). Animals were fed Harlan Rodent Chow 2016 and water was available at all times.

Hamster Estrous Cycles

All hamsters showed two consecutive 4-day estrous cycles as determined by a positive test for sex behavior (the lordosis posture) with a sexually-experienced male hamster one hour before the onset of the dark period on day 4, termed 'proestrous' in rats, and in this experiment, the 'periovulatory day.' The next day, estrous cycle day 1, is characterized by a copious vaginal discharge, high levels of agonistic behavior and is termed the 'postovulatory day.' The next day is termed 'follicular 1.' The next day is 'follicular 2,' the day of peak vaginal marking.

Experiment 2.1: Characterization of Food Hoarding Throughout the Light:Dark Cycle and Over the Estrous Cycle

Our aim was to document food hoarding and food intake every 6 h over the estrous cycle in order to determine whether our female laboratory hamsters, like wild hamsters, increase hoarding near the onset of the dark period, and to determine whether food hoarding varies over the estrous cycle in the absence of males under food restriction and ad libitum-feeding.

Eleven adult, estrous-cycling female hamsters, 110-160 g in body weight, were acclimated to their home cage and trained to recognize food in the food boxes attached by

plastic Habitrail tubing to their home cages. No males were present in the room during this experiment. A pre-weighed amount of food was placed in the food box (600-800 g) on the first day, and no food was placed in the home. Thereafter, the females' body weight and the amount of food remaining in the food box and the amount of food in the home were measured every 6 h for 6 days. At the end of weighing food and hamsters, all food was removed from the home, and pre-weighed food was placed in the food boxes. Food hoarding was the amount of food found in the home, or if any of the food was soiled, food hoarding was calculated as the amount of food in the food box subtracted from the amount in the food box 6 h earlier. Food intake was the amount of food remaining in the food cage and in the home subtracted from the amount of food in the food box 6 h earlier. Half of the hamsters were restricted to 75% of their ad libitum food intake and the other half remained on ad libitum food rations for one week. Two hamsters were removed from the study because they began to show irregular estrous cycles. The experiment was repeated, measuring body weight, food intake and food hoarding every 6 hours for 6 days. The animals were placed on ad libitum feeding for 2 months, then all of the previously food restricted females were fed ad libitum, while the previously ad libitum-fed were food-restricted for one week, and the experiment was repeated again. Together there were 9 females in the food restricted and ad libitum-fed groups.

Estrous cyclicity was determined by a 5-min lordosis test that occurred during the dark period, and females that showed lordosis were designated as periovulatory females (day 4 of the estrous cycle). Testing began for all females on the same date, and the

females were not synchronized with regard to the day of estrous. Thus, about one fourth of the females started the experiment on day 1 of the estrous cycle whereas others started on days 2, 3 or 4.

Experiment 2.2: Sex and Ingestive Behaviors Over the Estrous Cycle During Food Scarcity and Abundance

Our aim was to see whether energy restriction unmasks the effects of periovulatory hormones on hunger motivation. If the role of ovarian hormones is to divert attention toward sexual stimuli and away from food stimuli, females living in energy abundance might be expected to show little or no change in food hoarding over the estrous cycle, whereas females living in energy deficit would be expected to show more dramatic fluctuations in hoarding over the estrous cycle. More precisely, we predicted that 1) food hoarding would be lower at the time of ovulation when circulating estradiol levels are highest, 2) food hoarding would be maximal when circulating estradiol levels are low, and 3) food hoarding would be high in food-restricted females except on the periovulatory day when females would show a preference for males over food, producing a marked fluctuation in these behaviors over the estrous cycle. We also tested whether fluctuations in hoarding over the estrous cycle would be exaggerated by restricting the time available for hoarding.

The Preference Apparatus

To examine the female hamsters' motivation to engage in either ingestive or sex behaviors, the hamsters were tested in a specially-designed preference apparatus described previously (Schneider et al., 2007). Each apparatus consisted of a home cage connected to both a food source box and third box that contained an adult male, and all three boxes were connected to each other by plastic tubes. Each female hamster was housed in the home cage made from opaque, Nalgene cages (31 × 19 × 18-cm) lined with fine ground wood shavings with a specialized door at one end that was kept closed when the animal was not being trained or tested. During training or testing, the door led via plastic tubes to two separate chambers. The tube from the home cage was approximately 134 cm in length and led vertically to two horizontal tubes 40-50 cm in length, connected in a T configuration. One horizontal tube was connected to a clear plastic chamber (the food box) containing a weighed amount of hoardable pellets. Hoardable pellets were made from standard laboratory chow pellets that had been cut into 2 cm pieces, a size that hamsters readily fit into their cheek pouches and that allow the females to fit through tubes with their cheek pouches full. The second horizontal tube was connected to a clear, Plexiglas cage (27 × 20 × 15-cm) (the male box) containing an adult, sexually-experienced male but did not contain food or water.

Acclimation to the Home Cage

Animals were placed in the home cage with fine wood chip bedding, cotton nesting material, food, and water, and allowed to live there for four to seven days with the door closed and no access to the male or to the food boxes. This allowed them to be

acclimated to their home cage and reduced any tendency to sleep in or carry bedding or food to the other chambers.

Training in the Preference Apparatus

Once acclimated to the home cages, hamsters were trained to expect food in the food box and males in the male box. Training sessions (like testing sessions) began within one hour of the onset of the dark phase of the photoperiod and extended 1 to 2 hours into the dark period, because this is near a time when hamsters are active in nature (Gattermann et al., 2008) and overlaps with the nocturnal hoarding period in our laboratory (result of Experiment 2.1). Each training session with access to the food box lasted 90 min and was given over 4 sequential days, encompassing all days of the estrous cycle. Females were allowed to discover the food box and to keep all of the food that they hoarded from the food box in their home cage. Each training session with access to the male box occurred on two consecutive days, on days 3 and 4 of the estrous cycle (i.e., follicular 2 and the periovulatory day). During the training session that occurred on day 4 of the estrous cycle, the day of lordosis, the females were allowed access to the male box, which contained an adult sexually-experienced male. The male and female were allowed to interact under close supervision by the experimenter for 2-5 min so that the females received anogenital licks and ectopic mounts but no intromissions or ejaculations. During the training session that occurred on day 3 of the estrous cycle (i.e., follicular 2), the females were allowed to enter the male cage with an unrestrained male (females cannot be impregnated on this day of the cycle) for 2-5 min or until a fight broke out, after

which, both were returned to their respective cages. Females that receive this training reliably leave their home and either hoard food or interact with a male (Schneider et al., 2007).

Testing in the Preference Apparatus

Preference testing began within 1 hour of the onset of the dark phase of the photoperiod. To start the test, the door to the vertical tube was opened and the female subjects were allowed access to both the food and male boxes for a total of 90 min. During the first 15 min, all female behaviors and location were recorded every 5 sec by an experimenter. The experimenter recorded vaginal marking, flank marking, hoarding, and eating. At the end of 15 min, the experimenter stopped recording, but female still had access to both the food box and the male box for 75 min. At the end of the final 75 min (90 min total), access to the food and male box ended, and the weight of the food in the apparatus (the home cage and the food box) was measured and recorded. This gave a measure of food hoarding and eating within the context of an available mating partner.

Procedures

Twenty-four female hamsters 100-150 g in body weight were acclimated and trained in the preference apparatus as described in the general methods. Baseline food intake was measured for 4 days by giving a weighed amount of food in the home cage and weighing the food remaining (minus pouched and spilled food). The hamsters were divided into 3 groups that did not differ in body weight or food intake: Group 1 had

unlimited access to the food box and were given as much food as they cared to eat each day ("Ad libitum time and food"), Group 2 was given limited food but unlimited time of access to the food box ("Food-limited"), Group 3 was limited to only 90 min access to the food box, but was not limited in the amount of food ("Time-limited"). More detail for each treatment group is given below.

Ad libitum-time and Food Group

At the start of the 8-day treatment period, 8 control hamsters were allowed access to the food box containing at least 200-250 g of food for 24 h a day. They were allowed to hoard as much as they wanted, and their access remained unlimited for 8 days. The food in the food box was replenished as necessary.

Food-limited Group

Each hamster in the Food-limited group was given 75% of her own average daily baseline food intake (4.5-8.25 g). This food was placed in the food box, and the females had unlimited access to the food box for 8 days. In the Food-limited group, the pre-weighed food was provided in two daily rations placed in the food box at random times throughout the day so as not to entrain activity rhythms (Rusak et al., 1988). Each ration was provided at least 6 h later than the last ration. Hamsters in this group tended to visit the food box many times a day, but only received 75% of their daily baseline intake every 24 h. After each test period, food intake was measured and the females were provided only with enough food to remain restricted at the same level as before the start of testing.

Time-limited group

The hamsters in the Time-limited group were given 200-250 g of food in their food box each day, but were only allowed to visit the food box for 90 min each day, beginning at the onset of the dark phase of the photoperiod, for 8 days. They were allowed to keep all the food that they hoarded from the food box to the home cage each day. If a hamster failed to hoard any food, 20 g of food was placed in its home cage that day.

After the 8 days of the above treatments, all hamsters were tested in the preference apparatus with access to both the food box (containing a pre-weighed amount of food) and the male box for 90 min on each of the days of the estrous cycle, and thus, each animal was tested once per day on 4 consecutive days. Testing was scheduled to begin around the start of the dark phase of the photoperiod because it has been reported that wild hamsters hoard daily at this time, and a nocturnal hoarding rhythm in our laboratory hamsters was confirmed in Experiment 2.1 when food hoarding was measured around the clock every 6 h over the estrous cycle.

Estrous cycles were not synchronized, so testing began on each of the 4 estrous cycle days for roughly one quarter of the hamsters in each group. Hamsters were tested in counterbalanced order with regard to their experimental group so that no one group would be tested at a particular time of day relative to the other groups. The preference test occurred as described in the general methods.

Repeated measures analysis of variance (ANOVA) was used to analyze changes over the estrous cycle in the amount of food hoarded and eaten, male preference (time with male – time with food)/total time, and the number of vaginal and flank marks. Differences among the groups on each day of the estrous cycle were analyzed using one-way ANOVA followed by Duncan's Multiple Range Test when main effects were significant. Differences were considered statistically significant if P was less than 0.05.

Experiment 2.3: Effects of Exogenous Estradiol and Progesterone in Food Scarcity and Abundance

Our aim was to determine whether the hormone treatment known to bring ovariectomized (OVX) hamsters into estrous also decreases food hoarding and/or food intake. We hypothesized that one role of ovarian hormones is to attenuate the urge to hoard food and increase the urge to mate. If so, removal of ovarian hormones by OVX would increase food hoarding, and treatment of OVX females with the hormones characteristic of estrus would do the opposite, and furthermore, the conditions of energy scarcity would exaggerate the difference in hoarding between OVX-vehicle and OVX + hormone groups. Thus, we suggested that food restriction would unmask the effects of exogenous hormones on appetitive aspects of ingestive and sex behavior. This time, instead of comparing females at different stages of the estrous cycle, we compared OVX females treated with estrous-inducing doses of estradiol benzoate (E) and progesterone (P) or the oil vehicle. If our hypothesis were supported, we would expect to see larger differences in food hoarding between OVX and OVX-E+P groups when the females were

food-restricted than when they were allowed to eat ad libitum. In addition, we asked whether the effects of estradiol are dose-dependent or threshold effects by varying the dose of estradiol and keeping the dose of progesterone constant.

Doses of Estradiol and Progesterone

As a first step toward understanding the role of ovarian steroids, we used a hormonal treatment regimen known to induce estrous behavior in OVX Syrian hamsters, i.e. a subcutaneous (s.c.) injection of estradiol 48 h before testing followed by an injection of progesterone 6 h before testing) (Feder et al., 1974; Jones et al., 2002). If our hypothesis about the role of ovarian steroids in decreasing the urge to forage while at the same time increasing the urge for mating were supported, it would be expected that experimental regimens that are most effective in inducing sex behavior would also reduce food hoarding.

In addition, we varied the dose of estradiol to determine whether this hormone produced a linear dose-response or threshold effect on food hoarding and preference for males. The different doses of estradiol produce circulating levels within the range of hamsters on follicular day 1 (the low dose 1.5 μg), the follicular day 2 (the medium dose, 2.5 μg) or at a superphysiological level (the high dose, 20 μg) (Baranczuk and Greenwald, 1973; Jones et al., 2002).

Forty-four female Syrian hamsters 105-145 g in body weight were deeply anesthetized using sodium pentobarbital (80 mg/kg; Sigma Aldrich, St. Louis, MO) and bilaterally ovariectomized through bilateral flank incisions closed with suture (muscle

incision) and wound clips (skin incision). An analgesic (Metacam, Boehringer Ingelheim Vetmedica, Inc. St. Joseph, MO, 0.06 ml) was given at the time of anesthesia to minimize pain and discomfort after surgery. After recovery, hamsters were randomly placed into treatment groups that did not differ significantly in body weight and would receive different doses of estradiol (Sigma Aldrich, St. Louis, MO) dissolved in the canola oil vehicle.

Experiment 2.3 used a 2 by 4 factor design, with hamsters receiving one of 2 energy treatments (ad libitum-fed or food restricted) and within each of these energy treatments, 4 subgroups received one of 4 hormone treatments (one group was vehicle-treated, the other 3 groups were all progesterone-treated, but separate groups received one of 3 doses of estradiol). In total, there were 8 groups of 5-6 hamsters per group.

Daily food intake was measured for 4 days to find each hamster's individual average daily food intake. Then, half were fed either ad libitum or restricted to 75% of their ad libitum daily food intake, separated into two portions, one of which was provided every 12 h. Food restriction or ad libitum-feed was scheduled to continue for 12 days (with testing on days 4, 8 and 12) and then the previously food-restricted hamsters were returned to ad libitum feeding to be tested at 4 and 8 days after re-feeding. The only exception was that if food restricted females that lost body weight failed to show lordosis in response to a male, she was immediately returned to ad libitum feeding and test 4 and 8 days later. All Ad libitum-fed and restricted groups received either vehicle or one of the doses of estradiol 48 h before testing, and progesterone six h before every behavioral test.

Beginning 4 days after the start of food restriction, all hamsters (ad libitum-fed and food-restricted) were tested in the preference apparatus in counterbalanced order every 4 days while they remained on their respective diets. Hamsters were injected s.c. with their designated dose of estradiol at 0800 h and placed back into their home cage.

Forty-eight h following the injection of estradiol, all hormone-treated hamsters received 500 µg of progesterone (Sigma Aldrich, St. Louis, MO) dissolved in canola oil whereas the vehicle-treated group received the canola oil vehicle at the same volume. Six ± 1 h later, hamsters were tested in the preference apparatus as described in the general methods with some minor changes. Just before the start of the test, an adult male hamster was placed into a wire cage inside the male box and a preweighed amount of food (150 ± 10 g) was placed in the food box. After the door to the home cage was opened, the female's location and behaviors were observed and recorded every 5 sec for 10 min. Females that reached the male box and made tactile or oral contact with the male (through his wire cage) were given light stroking to their flanks by the experimenter. If the female did not show lordosis in the presence of a restrained male coupled with gentle flank stimulation by the end of the 10-min testing period, the male was released from his cage and allowed to mount the female. Once the male was removed, the experimenter recorded the latency to mount, but not lordosis duration. At the end of the 10-min period of recording the female behavior, the male was secured in the protective box and females continued to have access to the food or male boxes for an additional 80 min. After the completed test (90-min in total), food in the various compartments was measured to determine the amount of food hoarded and eaten, and the male and female hamsters were

returned to their home cages. All females received a preweighed amount of food and the amount of food remaining was measured the next day prior to the beginning of testing.

Differences among the groups on days 4 and 8 after the start of food restriction and on day 4 after re-feeding were analyzed using two-way ANOVA followed by planned comparisons. Differences were considered statistically significant if P was less than 0.05.

Experiment 2.4: Effects of Estradiol or Progesterone Alone on Appetitive Behavior

The results of Experiment 2.1 show that food restriction alone increases food hoarding, but has little or no influence on the pattern of food hoarding over the estrous cycle when no male hamsters were present (Figure 2.1). When no males were present, food-restricted females showed higher levels of food hoarding during the dark phase of the photoperiod and these levels did not fall on the night of estrous. In Experiment 2.2, however, when males were present, food hoarding was increased by food restriction on all nights of the cycle, expect the night of estrous. In Experiment 2.3, food-restricted induced increases in food hoarding were attenuated by estradiol treatment in ovariectomized females when males were present. Thus, we hypothesized that hormones of the estrous cycle influence food hoarding by increasing sexual motivation, not by direct effects on food hoarding. To test this hypothesis we compared the effects of estradiol with and without progesterone in females tested first in the presence of males and then in the absence of males.

32 female Syrian hamsters (142 ± 15 g body weight) were trained to the preference apparatus containing an adult male stimulus hamster, OVX female stimulus hamster, and a food source as described in the general methods. 24-h food intake was measured for a minimum of 4 days during training. After training, subject females were deeply anesthetized using 80 mg/kg of sodium pentobarbital (Ovation Pharmaceuticals, Inc., Deerfield, IL) and OVX through bilateral flank incisions closed with suture (muscle incision) and wound clips (skin incision). An analgesic (Metacam, 0.06 ml; Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) was given at the time of anesthesia to minimize pain and discomfort after surgery. At least one week after surgery, hamsters were randomly placed into treatment groups that did not differ in body weights. Groups included 1.) estradiol capsule and oil injection; 2.) estradiol capsule and progesterone injection; 3.) cholesterol capsule and oil injection; 4.) cholesterol capsule and progesterone injection. Afterwards, hamsters were restricted to 75% of their pre-ovariectomy food intake until testing. No ad libitum-fed hamsters were used in this experiment because we wanted to test the effects of ovarian hormones on sexual and ingestive behaviors and ad libitum-fed females hoard little food and they spend nearly the entire test with a male (Klingerman et al., 2010, Klingerman et al., 2011). After 4 days of food restriction, hamsters were implanted with Silastic capsules containing either crystalline estradiol (n=16) or the cholesterol vehicle (n=16) under light anesthesia into the subcutaneous interscapular region. Hormone capsules were made using Silastic tubing (No. 602-235, 0.058" i.d., 0.077" o.d., 0.009" wall thickness, Dow Corning Co.). Capsules were a total of 12 mm in length with silicone sealant placed into the tubing at 2

mm on each end, for a total of 8 mm of estradiol or cholesterol treatment.   This length of estradiol-containing capsule has been shown to induce vaginal marking from 6 to 30 hours after implantation (Lisk and Nachtigall, 1988). Capsules were incubated overnight at 37°C in 0.9% NaCl to prime the capsules for immediate release following implantation.

By providing the subject females a choice between visiting a male and a OVX stimulus hamster we could determine whether the experimental effects on preference were specific to males, or to adult conspecifics in general. If, for example, subject females showed an equal preference for OVX females as intact males, this would suggest decreased interest in sex and more interest in other types of social interaction (e.g., defensive aggression) (Klingerman et al., 2010, Schneider et al., 2007, Klingerman et al., 2011).

48 h after hormone implantation (6 days of food restriction), females were injected with either progesterone (500 µg) or its vehicle, canola oil in a similar amount and tested in the preference apparatus 6 h later. Behavioral observations including vaginal and flank marking, food hoarding, and eating and the subject females' location were recorded every 5 sec by an observer for 15 min. Hamsters given an estradiol capsule followed by a progesterone injection were tested for the lordosis reflex by an experimenter that provided light stimulation to the females' flanks after she entered into the male box. After 15 min of observation, stimulus females continued to have access to the apparatus for an additional 75 min (total 90 min). After the test, food in the food and home cages was weighed and the amounts hoarded and eaten were calculated.

After being exposed to the preference apparatus in the presence of male and OVX female stimuli for 90 min, stimulus animals were returned to their respective cages and the amount of food hoarded into the home cage and the amount of food eaten was determined. Food was replenished into the food box and immediately afterwards, subject females were given an additional 90 min to hoard and eat food in the absence of male and OVX female stimuli. The amounts of food hoarded and eaten were determined.

Subject females were given a similar behavior test in the preference apparatus in the presence and absence of male and OVX female stimuli for 90 min after a prolonged period of food restriction, 10 days. After the last test, females were euthanized with an overdose of nebutal. Subcutaneous, visceral, gonadal, perirenal, and intraperitoneal fat pads were excised and weighed for comparison.

Results

Experiment 2.1: Daily Rhythms in Hoarding, Body Weight and Food Intake

Food Hoarding

Food hoarding progressively increased the first 2-3 times it was measured and then began to settle into a nocturnal pattern (Fig. 2.1, top). Only data from the last 4 of the 6 days were used for analysis. Both fed and food-restricted females that had no access to males hoarded more in the dark than in the light period ($P < 0.001$, Fig. 2.1, top). All daily peaks in food hoarding occurred during the dark, and all nadirs appeared in the light period, although small amounts of food were hoarded in the light period. Neither the highest hoarding score for each animal nor the lowest hoarding score for each animal

differed significantly over days of the estrous cycle. The group that was fed ad libitum decreased nocturnal food hoarding over time, whereas the food restricted females' nocturnal hoarding scores remained elevated and were significantly higher than those of the ad libitum-fed females on at least once time point every night ($P < 0.05$, Fig. 2.1, top).

Data were analyzed using 1-way ANOVA. Paired T-tests were used to compare food hoarding in the same treatment group in the presence and absence of social stimuli. Differences were considered statistically significant at $P < 0.05$.

Food Intake

Food intake showed a different pattern (Fig. 2.1, bottom). There was no significant difference between the amount of food eaten during the dark period and the amount of food eaten during the light period on 3 days of the estrous cycle, whereas very little food was eaten during the dark period of proestrous, the periovulatory period in the females fed ad libitum (Fig. 2.1, bottom). Food restricted females had elevated food intake on only two of the 24 time points measured ($P < 0.05$).

Body Weight

Body weight gain and loss showed cyclic fluctuations with regard to the light and dark phases of the photoperiod, with weight gain at night and weight loss during the light period, and no significant differences over days the estrous cycle (data not shown).

Experiment 2.2: Sex and Ingestive Behaviors Over the Estrous Cycle During Food Scarcity and Abundance

For all variables that were measured, the ad libitum-fed females did not differ significantly from the females that were time-restricted but given ad libitum access to food (Table 2.1), and thus, these two groups are combined into one group and named "Energy-abundant" in all graphs. The food-restricted females are referred to as "Energy-limited."

Food Hoarding

Food hoarding was significantly greater in Energy-limited compared to Energy abundant females on the postovulatory day and on follicular day 1 ($P < 0.009$ and 0.003 respectively, Fig. 2.2, top). Energy-limited hamsters showed a marked decrease in food hoarding on the night of follicular day 2 (the night of vaginal scent marking) to a level not significantly different from Energy-abundant hamsters (Fig. 2.2, top). On the night of proestrous, the periovulatory period, food hoarding was at its nadir but was still significantly higher in the Energy-restricted group compared to the Energy-abundant group because the latter group hoarded no food and was in persistent lordosis on the periovulatory day ($P < 0.04$, Fig. 2.2, top). In the Energy-limited group, repeated measures ANOVA revealed significant changes over the estrous cycle in food hoarding ($F(3,15) = 3.7$, $P < 0.04$, Fig. 2.2, top), and fluctuations over the estrous cycle in hoarding were not statistically significant in the Energy-abundant group (Fig. 2.2, top).

Food Intake

In contrast to food hoarding, there were no significant changes over days of the estrous cycle in 24-h food intake in either group or in the groups combined, and females that were Energy-limited did not differ from Energy-abundant in food intake during testing (Fig. 2.2, bottom).

Male Preference

In the Energy-limited, but not Energy abundant females, there were significant changes over the estrous cycle in the preference for males (time with males – time with food)/total time (Fig. 2.3), and the Energy-restricted hamsters showed significantly lower preference for males than Energy-abundant on all days of the estrous cycle ($P < 0.004$, 0.001, 0.01 and 0.003 for the four days shown in Fig. 2.3).

Lordosis and Scent Marking

There were no significant differences between Energy-restricted and Energy-abundant in lordosis duration (measured on the periovulatory day) nor vaginal nor flank marking (measured on Follicular 2, Fig. 2.4). In Energy-abundant but not restricted females, vaginal scent marking changed significantly over the estrous cycle with the highest level of vaginal scent marking on follicular day 2 of the estrous cycle, the day before mating and ovulation ($F(3,15) = 4.3$, $P < 0.01$, Table 2.1) and significant changes over the estrous cycle in flank marking ($F(3,12) = 4.4$, $P < 0.01$, Table 2.1).

Experiment 2.3: Effects of Exogenous Progesterone and Estradiol in Food Scarcity and Abundance

Food Hoarding

First, the data were analyzed with the hoarding scores of hamsters receiving different doses of estradiol divided into separate groups (Fig.2.5, top). With regard to food hoarding on day 4 of food restriction, two-way ANOVA showed a significant main effect of hormone treatment ($F(3,38) = 3.0$, $P < 0.04$). Planned comparisons showed the hoarding scores of food-restricted, vehicle-treated hamsters were significantly higher than those of each of the food-restricted, E+P-treated groups ($P < 0.01$, 0.05, and 0.03 for 1.5, 2.5 and 20 μg respectively, Fig. 2.5, top).

There were no significant differences in hoarding among the three E+P-treated groups (Fig. 2.5 and Table 2.2), and food hoarding was not significantly correlated with estradiol doses between 1.5 and 20 μg, and so these groups were collapsed into one hormone-treated group. When hoarding on day 4 of restriction was analyzed this way, there was a significant main effect of diet ($F(1,40) = 7.7$, $P < 0.01$), hormone treatment ($F(1,40) = 5.4$, $P < 0.02$) and a significant interaction ($F(1,40) = 4.3$, $P < 0.04$), reflecting the fact that restriction-induced hoarding significantly increased in the OVX vehicle-treated, but not in the OVX E+P hamsters ($P < 0.01$) (Fig. 2.5, bottom). On day 8, the main effect of restriction was significant ($F(1,40) = 11.54$, $P < 0.002$) but the main effect of hormone treatment was not, and both the vehicle and E+P-treated hamsters showed significant restriction-induced food hoarding ($P < 0.04$ and 0.003 respectively). In particular, the food-restricted, E+P-treated hamsters had high levels of food hoarding on

day 8 compared to ad libitum-fed, E+P-treated hamsters ($P < 0.05$), while they had low levels of food hoarding on day 4 of restriction and on day 4 after re-feeding (Fig. 2.5, bottom right). On days 4 and 8 after re-feeding, there were no significant main effects or interactions, although inspection of the rank order of the means shows that the vehicle-treated, food-restricted group was the last to return to baseline hoarding levels (Fig. 2.5, bottom left).

Male Preference

When we calculated the preference for males vs. food ((time with male – time with food)/total time), the different estradiol dose groups did not differ from each other and estradiol dose was not significantly correlated with male preference at 4 and 8 days after restriction (Table 2.3). The 3 groups treated with different doses of estradiol were collapsed into one E+P-treated group for days 4 and 8 after restriction. On day 4 of restriction, there was no significant main effect of energy availability on male preference and a significant main and of hormone treatment on male preference ($F(1,42) = 8.86$, $P < 0.05$), and planned comparisons showed that food-restricted, vehicle-treated females had a significantly lower male preference than did the food-restricted, E+P-treated females ($P < 0.008$) and there were no other significant differences (Fig 2.6). On day 8 of restriction, the main effect of diet ($F(1,42) = 14.71$, $P < 0.001$) and hormone treatment ($F(1,42) = 11.06$, $P < 0.002$) were significant (Fig. 2.6). Both the vehicle-treated and E+P-treated hamsters showed significant restriction-induced decrease in male preference (both $P < 0.04$).

To analyze male preference on days 4 and 8 after re-feeding, estradiol dose groups were not combined because there were significant differences between the 20 μg group and the other groups and male preference was significantly correlated with estradiol dose ($P < 0.05$). On day 4 after re-feeding, the main effect of hormone treatment and the interaction were significant for male preference ($F(1,42) = 12.9, 3.32$, $P < 0.0001, 0.03$ respectively), whereas the effect of diet was not significant. In the previously food-restricted, vehicle-treated females, preference for males was significantly increased relative to ad libitum-fed females ($P < 0.001$). In the food-restricted, E+P-treated females, male preference increased toward the levels of the ad libitum-fed females after 4 days of re-feeding (Fig. 2.6).

On day 8 after re-feeding, the main effect of diet and hormone treatment were significant for male preference ($F(1,42_{)} = 4.86, 13.66$, $P < 0.03, 0.0001$ respectively). In the previously food-restricted, vehicle-treated females, preference for males was significantly increased relative to ad libitum-fed females ($P < 0.002$), and in the food-restricted, E+P-treated females, male preference increased toward the levels of the ad libitum-fed females after 4 days of re-feeding (Fig. 2.6).

Hormone treatment significantly decreased time spent in the home cage. There was a significant main effect of hormone treatment on the preference for spending time in the home on all four days ($F(1,40) = 10.73, 5.99, 3.9$, and 5.7 and $P < 0.002, 0.02, 0.05$ and 0.02 for each time point respectively), with no significant dose-response relation between 1.5 and 20 μg E+P (data not shown). The ad libitum-fed, E+P-treated females showed a significantly lower preference for the home compared to ad libitum-fed,

vehicle-treated females at all time points ($P < 0.03$). The food-restricted, E+P and the food-restricted, vehicle-treated hamsters all showed similarly low preference for the home cage (data not shown), and these groups did not differ significantly.

Lordosis Duration

All E+P treated groups decreased lordosis duration at 8 days of restriction and increased lordosis duration at 4 days of re-feeding, as repeated measures ANOVA showed a significant change over time ($F(3,45) = 4.134$, $P < 0.01$) in the food-restricted but not in the ad libitum-fed group. After the restriction period extended to 8 days, even those females treated with E+P decreased lordosis duration (Figs. 2.7). For hamsters fed ad libitum or food-restricted, there were no significant differences among the hamsters receiving different doses of estradiol in lordosis duration on day 4 or 8 after re-feeding.

Only those hamsters treated with E+P showed lordosis. The frequency of ad libitum-fed females that showed lordosis in response to the restrained male and in response to the unrestrained male was not significantly different among the groups treated with different doses of E+P (Fig. 2.8). Food restriction for 8 days, but not 4 days significantly decreased the number of females that showed lordosis, and fewer females showed lordosis in response to the restrained male compared to the number that showed lordosis in response to the unrestrained male ($P < 0.05$). Almost all of the females failed to show lordosis at 12 days after the start of food restriction (Fig. 2.8). The frequency of females that showed lordosis in response to E+P in response to a male was significantly

lower after 8 days of restriction in the group treated with the middle dose of estradiol (2.5 μg) than the number that showed lordosis in the other two groups (Fig.2.8).

Experiment 2.4: Effects of Estradiol or Progesterone Alone on Appetitive Behavior

Food Hoarding

As we predicted, in the absence of males, food restriction increased food hoarding and none of the hormone treatments attenuated this effect (Figure 2.9, Bottom). Consistent with the results of Experiment 2.3, estradiol alone or estradiol plus progesterone decreased food hoarding in the presence of males in the early, but not the late stages of food restriction (Figure 2.9, Top).

After 6 days of food restriction, there was a main effect of hormone treatment on food hoarding, ($F(3,26) = 4.11$, $P < 0.01$) (Figure 2.9 top). Hamsters given progesterone hoarded significantly more food than all other groups in the presence of male and female stimuli ($P < 0.05$) (Fig. 2.9, Top) after 6 d of food restriction and 6 h after hormone injection. Also at this time, hamsters given E+P hoarded the least amount of food of all groups. In the absence of male and female stimuli, all females increased food hoarding and there was no significant difference among treatment (Fig. 2.9, Top).

After 10 days of food restriction and 6 h after hormone injection and in the presence of male and female stimuli, there were no longer any significant differences in the amount of food hoarded among groups, all females hoarded (Fig. 2.9, Top); however, hamsters given progesterone hoarded the most and hamsters treated with estradiol hoarded the least amount of food at this time point. Similar to 6 days of restriction, after

10 days of food restriction, in the absence of male and female stimuli, all females increased food hoarding and there were no significant differences among treatment groups (Fig. 2.9, Bottom).

Number of Vaginal and Flank Marks

There was a significant main effect of hormone treatment on vaginal marks after 5 days of restriction ($F(1,28) = 6.40$, $P < 0.02$) (Table 2.4). Hamsters implanted with estradiol vaginal marked significantly more than hamsters given vehicle 30 h after Silastic capsule implantation which corresponded to 5 days of food restriction ($P < 0.05$). There was also a significant main effect of hormone treatment on the incidence of vaginal marking 54 hours after estradiol or vehicle capsule implantation and 6 hours after progesterone or vehicle hormone injection which corresponded to 6 days of food restriction ($F(3,26) = 3.19$, $P < 0.04$). Post hoc comparisons revealed that hamsters given estradiol produced significantly more vaginal marks compared to all other groups at this time point ($P < 0.05$). There was no difference in the number of flank marks produced by any group at any time point of food restriction or hormone treatment.

After 10 days of food restriction, there was no longer a main effect of hormone treatment on vaginal marks.

Time Spent with Social Stimuli

There was significant main effect of hormone treatment on the amount of time subject females spent with the male stimulus hamster after 6 d ($F(3,26) = 5.13$, $P < 0.006$)

and 10 d ($F(3,25) = 3.67$, $P < 0.03$) of restriction (Fig. 2.10). Post hoc analysis revealed that after 6 d of food restriction (Fig. 2.10, Top), subject females treated with estradiol + progesterone spent significantly more time with a male compared to subject females treated with progesterone alone or vehicle, but not compared to subject females given estradiol alone ($P < 0.05$). Similar results were obtained after 10 d of food restriction (Fig 2.10, Bottom), with females given estradiol + progesterone spending more time with a male compared to females give progesterone or vehicle, but not females given estradiol alone ($P < 0.05$).

In contrast to the male, there was no main effect of hormone treatment on the amount of time subject females spent with OVX stimulus female hamsters after 6 d or 10 d of food restriction (Fig. 2.10).

Time Spent with a Food Source

When male stimulus animals were present, there was a main effect of hormone treatment on the amount of time subject females spent with a food source during testing after 6 d ($F(3,26) = 3.14$, $P < 0.04$), but not 10 d of food restriction (Fig. 2.10). Post hoc analysis revealed that after 6 d of restriction, females given progesterone alone spent significantly more time with food during testing than females given estradiol + progesterone ($P < 0.05$).

Time Spent in the Home Cage

There was no main effect of hormone treatment on the amount of time subject females spent in the home cage after 6 d or 10 d of food restriction.

Amount of Food Consumed

The amount of food consumed during the 90 min test in the presence of male and female stimuli did not differ among groups at any time point measured (Table 2.5). Similarly, when tested in the absence of male and female stimuli, subject females never differed in the amount of food consumed during the 90 min test.

Body Weight

There was no significant difference in body weight among treatment groups at the start of the experiment. At the end of the experiment (10 days of food restriction), there was no significant difference in body weight or change in body weight among the hormone-treated, food-restricted groups (Table 2.5).

Fat Pad Weights

Hormone treatment had no significant effect on the wet weight of fat pads excised from the hamsters in this experiment (Table 2.6). However, females treated with estradiol or E+P had numerically lower unilateral subcutaneous and intraperitoneal fat and total visceral fat.

Discussion

These experiments demonstrate that ovarian hormones increase appetitive sex behaviors and decrease appetitive ingestive behaviors, but hormonal effects differ as a function of the availability of food and potential mating partners.

First, when males were absent and energy was abundant, food hoarding was highly nocturnal but did not vary over the estrous cycle, and food restriction did not exaggerate fluctuations over the estrous cycle in food hoarding (Experiment 2.1). Next, when access to adult males was coupled with limited energy availability, nocturnal food hoarding decreased and sexual motivation increased at the estrous period in association with high circulating levels of estradiol, but this effect was masked in females with unlimited energy availability (Experiment 2.2). Third, exogenous treatment with ovarian hormones delayed the onset of food restriction-induced hoarding and decreased the preference for males over food at 4 and 6 days after restriction but failed to do so after 8 and 10 days of food restriction (Experiments 2.3 and 2.4). Last, when the previously food-restricted females were returned to ad libitum food intake, ovarian hormone-treated females rapidly decreased hoarding and increased male preference relative to oil-treated females (Experiment 2.3). These results are consistent with the idea that the hormones of the estrous cycle modulate ingestive and sex behaviors according to the availability of energy and potential mates. This role of estradiol was not obvious in previous experiments because ingestive behaviors were measured in females isolated from males and under conditions of artificial energy abundance and minimal energy demands. The present experiments are rare in that they examined the choice between food and sex under conditions that mimic important aspects of the natural habitat. When females were

energetically challenged and offered a choice between food and males, energy restriction unmasked the function of ovarian hormones, i.e., to ensure that females risk an investment in reproduction during the fertile period despite the conflicting motivation to hoard food in anticipation of future food shortages.

In Experiment 2.1, the nocturnal pattern of food hoarding was consistent with previous experiments in which male Syrian hamsters hoarded more during the dark phase of the photoperiod, but females were not included in previous studies (Charlton, 1984; Toates, 1978; Waddell, 1951). Other investigators have examined food hoarding over the estrous cycle in singly-housed female hamsters, but only for 30 min per day at the onset of the dark period, and in these studies, females showed a nadir in food hoarding on the night of proestrous (Estep et al., 1978). The present study showed that when females were housed singly with ad libitum access to food, day-night differences in hoarding dwarfed any differences among days of the estrous cycle (Fig. 2.1). Food restricted, singly-housed females in Experiment 2.1 did not show the marked fluctuation in food hoarding compared to those that had the option of visiting a male in Experiment 2.2. The circadian pattern of food intake, in contrast to food hoarding, was not strictly nocturnal, as has been shown previously. Syrian hamsters eat small meals throughout the light and dark periods, consuming between 40-45% of their food during the light period (Schneider et al., 1986; Silverman and Zucker, 1976; Zucker and Stephan, 1973).

In Experiment 2.2, energy-restricted female hamsters with access to males showed marked fluctuations in food hoarding over the estrous cycle compared to ad libitum-fed females (Fig. 2.2) and time-limited hamsters fed ad libitum (Table 2.1).

Decreases in food hoarding at proestrous have been reported in both Syrian hamsters and rats when hoarding was measured at one time point on every day of the estrous cycle in the absence of males (Estep et al., 1978; Herberg et al., 1972), but our experiment makes the important distinction that energy restriction (but not time restriction) increases food-related behaviors on nonestrous days and decreases food-related behaviors during the periovulatory period, thus creating marked fluctuations in hoarding over the estrous cycle (Fig. 2.2).

Appetitive, but not consummatory behaviors were affected by mild food restriction. Appetitive behaviors are defined as those involved in the motivation to seek out and come in contact with food or mates and are, to some degree, independent of the consummatory acts of eating or copulating (Ball and Balthazart, 2007; Beach, 1976; Craig, 1917; Everitt, 1990; Lorenz, 1950; Sherrington, 1906). Depending on the species under study, appetitive behaviors are expressed as specific paracopulatory behaviors that occur before or after copulation (vaginal marking is one example), or as other expressions of sexual motivation that can overlap in time with copulation, such as the amount of time spent with males versus food. The distinction between appetitive and consummatory behaviors can be useful when these behaviors are controlled by different mechanisms or are differentially responsive to environmental stimuli (reviewed by Ball and Balthazart, 2007). For example, in Experiment 2.1, the appetitive ingestive behavior, food hoarding, increased markedly in food-restricted females (Fig. 2.2) whereas the consummatory ingestive behavior, food intake did not increase at any time during the study (Fig. 2.2). In a similar vein, the appetitive sex behaviors, male preference (Fig. 2.3) and vaginal

scent marking (Table 1), were decreased in food-restricted females without significant effects on consummatory sex behavior, lordosis duration (Fig. 2.4). These results confirm previous results that demonstrate separable mechanisms governing appetitive versus consummatory sex and ingestive behavior in rats and Syrian hamsters (Ammar et al., 2000; Buckley and Schneider, 2003; Schneider et al., 2007), and ingestive behavior in Siberian hamsters (Bartness, 1997), and suggest we should examine the mechanisms whereby hormones prioritize the motivations that control potentially conflicting behaviors.

In contrast to food availability, time availability had no effect when hamsters had ad libitum food intake in Experiment 2.2. We had postulated that limited time availability might increase food hoarding, and that ovarian hormones might ameliorate them. In the wild, Syrian hamsters are thought to be subject to environmental pressures that might limit the time they spend outside their burrows to particular times of day, e.g., pressures such as nocturnal predators (e.g., carnivorous birds, reptiles or mammals), intense mid-day heat, or low nighttime ambient temperatures (Gattermann et al., 2008). If time for daily hoarding is limited by these pressures, an important role for periovulatory increases in circulating estradiol might be to divert energy toward courtship and mating even though this might mean missing the day's only opportunity for foraging and food hoarding. If time is a limiting factor that interacts with ovarian hormone levels, it would be predicted that hamsters with limited time for both mating and hoarding would show more dramatic fluctuations over the estrous cycle in hoarding than those with unlimited time. To the contrary, this hypothesis was not supported, since time-restricted

females with unlimited access to food did not differ from females with unlimited time and food in any variable measured (Table 2.1). It was noted that the time-limited females hoarded significantly more than the ad libitum-fed group prior to testing and during training, when subject females did not have access to males (data not shown). Time-limited females learned to use their limited hoarding time to gather food during the training period, yet they did not retain this behavior once males became available. Instead, they chose to spend their time in close proximity to males, perhaps because there was no energetic consequence to this decision (the experimenter provided food after the end of the test). The lack of fluctuation in hoarding over the estrous cycle in the time-limited group reinforces the notion that ovarian steroids interact with energy availability to determine the choice between food and sex.

Food hoarding in restricted females fluctuated in association with well-known changes in circulating estradiol and progesterone. The amount of food hoarded was lowest during the proestrous periovulatory period, subsequent to increases in circulating estradiol concentrations in female Syrian hamsters (Baranczuk and Greenwald, 1973). The amount of food hoarded increased the day after ovulation (when circulating progesterone levels increase), remained high during follicular day 1 (when both progesterone and estradiol are at their nadir), began to decline on follicular day 2 (when estradiol concentrations rise). To test whether ovarian hormones account for these fluctuations across the estrous cycle, the effects of exogenous estradiol and progesterone on hoarding in food-restricted females were examined in Experiment 2.3.

In Experiment 2.3, in oil-treated, OVX controls, food restriction increased food hoarding, whereas treatment with E+P delayed the effects of food restriction on food hoarding (Fig. 2.5). This recapitulates the suppression of food restriction-induced increases in hoarding at estrous in Experiment 2.2, and is the first evidence that the hormonal regime routinely used in this field of research to induce estrous behavior decreases food hoarding. The importance of energy availability was underscored when previously food-restricted females were returned to ad libitum food access. In re-fed, OVX females, E+P treatment rapidly increased male preference (the preference for males over food, Fig. 2.6). Even in the vehicle-treated hamsters, return to ad libitum-feeding significantly increased male preference (Fig. 2.6), cautioning against assuming that increased male preference relative to food preference is a strictly “sexual” behavior.

When restriction was increased to 8 and 12 days, reproductive behaviors were inhibited, confirming previous research in Syrian hamsters (Morin, 1975; Morin, 1986; Schneider and Wade, 1989), including paracopulatory appetitive behaviors, such as preference for males versus food (Schneider et al., 2007). Experiment 2.3 showed that the longer the food restriction period, the more likely reproductive behaviors would be eliminated, even in animals treated with exogenous doses of estradiol and progesterone, and confirmed energetic effects on appetitive, rather than consummatory sex and ingestive behaviors. After the restriction period extended to 8 and 12 days, even those females treated with E+P decreased lordosis duration and male preference and increased food hoarding and time spent in the food box (Figs. 2.5-8). In addition, as food restriction became more severe, the number of females that failed to show the lordosis reflex during

testing increased significantly (Fig 2.8). This effect of restriction was significantly higher when the males were restrained than when the males had full access to the females and were able to make tactile contact, lick and mount the females (Fig. 2.8). These data show that the appetitive aspects of sex behavior, those that reflect sexual motivation, are more sensitive to energy availability than sexual performance. Similarly, the appetitive ingestive behavior, food hoarding, is more sensitive to energy availability than the consummatory ingestive behavior, food intake. This work might also be the first to document the ability of prolonged food restriction to negate the ability of exogenous E+P to suppress food hoarding (Fig. 2.5).

In the present study, energetic inhibition of sexual motivation likely involves changes in sensitivity to hormones, rather than changes in circulating concentrations of hormones, because all females in Experiment 2.3 showed inhibitory effects of food restriction even though they had the same exogenous doses of E+P as ad libitum fed females. Previous work has demonstrated that the levels of food deprivation that decrease lordosis duration in OVX females treated with E+P have measurable effects on estradiol receptor alpha immunoreactivity (ERαIR) while at the same time, increasing, not decreasing circulating concentrations of ovarian steroids (Li et al., 1994). In food-deprived females, ERIR decreases in the ventromedial hypothalamus and significantly increases in the medial preoptic area, the arcuate nucleus, and the posterior parvicellular paraventricular nucleus of the hypothalamus (Li et al., 1994). The relevance of these findings with regard to food deprivation-induced decreases in lordosis duration, the HPG system and estrous cyclicity have been noted (Wade and Jones, 2004), and now should be

explored with regard to food hoarding and other behaviors that reflect behavioral motivation rather than ability.

The relationship between food hoarding and estradiol dose was not linear at the doses used in our experiment. Although a dose-response relation between estradiol and lordosis duration has been shown in other studies with a different experimental design (Jones et al., 2002). This dose-response relation was not apparent in our experiments when females had a choice between food and males (Table 2.1). In the present experiment, all three doses of estradiol effectively delayed the effects of food restriction on food hoarding and the time spent with food when compared to females treated with the oil vehicle. Previous work showed that food hoarding increases with ovariectomy and during pregnancy along with rising levels of circulating progesterone in hamsters and rats (Bartness, 1997; Coling and Herberg, 1982; Fleming, 1978), and treatment with estradiol alone decreases food hoarding in rats (Coling and Herberg, 1982). Further testing is underway to differentiate between the role of estradiol, progesterone, or E+P, hormones with clear effects on body weight and food intake in hamsters and other species (Bhatia and Wade, 1989; Swanson, 1968; Zucker et al., 1972).

There was a discrepancy between the intact (Experiment 2.2) and ovariectomized females treated with E+P (Experiment 2.3) in the efficacy of food restriction. In Experiment 2, 8 days of restriction had no effect on lordosis duration or food hoarding in intact females during the periovulatory period (Figs. 2.2 and 2.4), whereas in Experiment 2.3, 8 days of restriction significantly inhibited lordosis duration and increased food hoarding (Figs. 2.5 and 2.7). Differences between experiments might be due to the

inability of E+P injections to duplicate hormonal fluctuations in intact females or to unknown differences between the animals used in the two experiments. Both groups of females were between 3 and 4 months of age and between 100-150 g in body weight.

Experiment 2.3 used the hormone regimen that has been most commonly used to induce estrous behavior, E+P, and showed, for the first time, that it significantly attenuated food hoarding and the preference for food under energetically demanding conditions (Figs. 2.5 and 2.6). Experiment 2.4 assessed the function of estradiol and progesterone on food hoarding separately. E+P as well as E alone attenuated food hoarding, whereas progesterone alone induced food hoarding (Fig 2.9). This energy-hormone interaction suggests a mechanism whereby the circulating concentrations of estradiol and progesterone that are required for the lordosis reflex can also attenuate the drive to hoard food. Estradiol, in particular, might interact with energy availability at the level of intracellular metabolism, and this interaction might involve fuel oxidation, lipolysis or both. White adipose tissue estrogen receptors mediate estradiol-induced decreases in lipoprotein lipase activity that are a prerequisite to lipid storage (Wade and Gray, 1979; Wade et al., 1985), and liver and skeletal muscle are likely sites for estradiol-induced increases in thermogenesis and fatty acid oxidation that is thought to account for decreases in body weight that cannot be accounted for by decreases in food intake (Jones et al., 1991; Schneider et al., 1986). Thus, one possibility is that estradiol produces increases in the flow of oxidizable fuels away from storage in adipose tissue and into tissue where they are oxidized, which, in turn, might generate signals in brain or periphery that lead to decreased motivation for eating and hoarding and more motivation

for reproductive activities. Although not significant, we find it interesting that food-restricted females given only 2 injections of progesterone in Experiment 2.4 had heavier visceral, intraperitoneal, subcutaneous, and gonadal fat pads whereas females treated with estradiol or E+P had the lightest fat pads compared to other treatment groups. It would be interesting to determine how moderate doses of cyclic progesterone, given for a longer period of time, would influence food hoarding, weight gain, and adiposity under our food restriction regimen.

Experiment 2.4 assessed the function of estradiol and progesterone on food hoarding separately. In the presence, but not in the absence of male conspecifics, treatment of ovariectomized females with E+P or E alone attenuated food-restriction-induced food hoarding, whereas treatment with progesterone alone increased food hoarding relative to all other groups (Fig 2.9). This energy-hormone interaction suggests a mechanism whereby the circulating concentrations of estradiol and progesterone that are required for the lordosis reflex can also attenuate the drive to hoard food when females have the option to visit a male during their 90-minute above-ground period.

One mechanism that might account for this effect is if sexual motivation is tonically inhibited during most of the estrous cycle by the mechanisms that increase the drive to hoard food. For example, endogenous cannabinoids or other putative orexigenic peptides might have inhibitory actions on the mechanisms that control sexual motivation, and these effects might be disinhibited by estradiol. This idea could be tested in future experiments by treatment of food restricted females with antagonists to cannabinoid receptors (or receptors for neuropeptide Y or ghrelin). If this hypothesis is correct,

treatment with antagonists to the cannabinoid receptors would induce male preference and decrease food hoarding even during days 1 and 2 of the estrous cycle. In support of this idea, in rats, treatment with an antagonist to the cannabinoid receptor decreases the latency to approach a male in ovariectomized females treated with subthreshold doses of estradiol and progesterone (Lopez et al., 2009).

These experiments do not address the metabolic stimulus involved. One possibility is that neuropeptide-steroid interactions are influenced by low availability of metabolic fuels. Estradiol, in particular, might interact with energy availability at the level of intracellular metabolism, and this interaction might involve fuel oxidation, lipolysis or both. White adipose tissue estrogen receptors mediate estradiol-induced decreases in lipoprotein lipase activity that are a prerequisite to lipid storage (Wade and Gray, 1979, Wade et al., 1985), and liver and skeletal muscle are likely sites for estradiol-induced increases in thermogenesis and fatty acid oxidation that is thought to account for decreases in body weight that cannot be accounted for by decreases in food intake (Schneider et al., 1986, Jones et al., 1991). Thus, one possibility is that estradiol produces increases in the flow of oxidizable fuels away from storage in adipose tissue and into tissue where they are oxidized, which, in turn, might generate signals in brain or periphery that lead to higher levels of motivation for reproductive activities. In these experiments, progesterone was only given 6 hours before behavioral testing. It would be interesting to determine how moderate doses of progesterone, given with and without estradiol for time periods that mimic pregnancy, would influence food hoarding, weight gain, and adiposity under our food restriction regimen.

The role of ovarian steroids, especially estradiol, in stimulating risky sexual behaviors, exploration and novelty has been discussed with regard to species from a wide array of taxa, including women. In wild animals, sex behavior incurs substantial risks, due to increased chance of predation, injury, sexually-transmitted disease, parasites, other diseases and the disruption of social hierarchies (Wallen, 2000). In the laboratory, when primates are housed in small enclosures, mating occurs throughout the menstrual cycle. In contrast, when sex behavior is studied in the contexts in which these behaviors occur in nature, it becomes apparent that the hormones of the ovulatory period are essential for overcoming the risks involved in sexual liaisons (Wallen, 2001). In field studies and in large seminatural habitats that allow individuals to escape or hide from their peers, primate sex behavior clusters in association with high circulating concentrations of estradiol. The unnatural, small, enclosed chambers used in the early years of primate laboratory research obscured the importance of estradiol in control of female sexual motivation. The present studies highlight energetic context as another important factor that can either mask or reveal the effects of ovarian hormones on both sexual and hunger motivation. The ability of estradiol to increase sexual motivation and reduce ingestive motivation were most apparent in the early stages of restriction and re-feeding, perhaps reflecting that reproductive success is increased when sex drive is immune to mild energetic challenges and when sex drive is quick to rebound as food becomes abundant. More detailed studies are under way to examine whether energy-restricted females will mate directly after meals or bouts of food hoarding, whether mated females will increase food hoarding in anticipation of pregnancy and lactation and whether males will

provision females with nuptial gifts of food (Schneider, unpublished observations). All females treated with E+P spent significantly less time in the home compared to vehicle-treated females in Experiment 2.3; consistent with the notion that estradiol is associated with increased exploration, novelty and risk-taking (reviewed by Fessler, 2003). This result, however, was not replicated in Experiment 2.4. These inhibitory effects of estrous levels of estradiol on hoarding and stimulatory effects on exploration and courtship might have functional significance at the onset of puberty, during adult estrous cycles, in the interim of parturition and the next pregnancy and during seasonal fluctuations in ovarian steroids. Hamsters are seasonal breeders and become gonadally regressed during the short days of winter. Decreases in ovarian steroids might play an important role in autumn increases and spring decreases in food hoarding (Lynch et al., 1973, Mull, 1970), and these changes might be imperative for optimizing reproductive success.

One implication of these results in relation to the increase in worldwide obesity in human beings is that ovarian hormones, as well as other so-called ‘satiety peptides,’ might only influence ingestive behavior temporarily under specific energetic contexts. Ovarian hormones might attenuate the appetite for food under moderate calorie restriction, but according to Figs. 2.5, 2.6, and 2.9 would not be expected to have significant effects on appetite or food hoarding under more extreme calorie restriction or under excessive energy abundance coupled with little or no energetic demand. It has been noted that some so-called ‘satiety’ peptides are less effective at curtailing food intake under prolonged energy restriction (Henry et al., 2001). The rise in obesity might, in itself, constitute evidence that endogenous satiety peptides are largely ineffective under

conditions of food abundance in organisms with minimal requirements for energy expenditure. If the adaptive function of estradiol and other anorectic hormones during millions of years of evolution was to temporarily decrease appetite in favor of sexual ventures, perhaps it is too much to expect of these hormones to maintain body weight within some healthy and fashionable range of our own modern invention.

Table 2.1

Preference behavior	Follicular Day 1		Follicular Day 2		Periovulatory Day		Postovulatory Day	
Number of Vaginal Marks								
Time-restricted	4.0	± 1.2	4.4	± 2.3	1.0	± 1.0	3.0	± 1.1
Ad libitum-time	3.6	± 2.5	10.6	± 3.1	0	± 0	2.6	± 1.6
Number of Flank Marks								
Time-restricted	2.8	± 1.9	4.4	± 2.9	0	± 0	0.2	± 0.2
Ad libitum-time	5.1	± 2.8	11.1	± 4.4	0	± 0	3.3	± 1.4
Food Hoarded (g)								
Time-restricted	48.7	± 40.7	12.4	± 11.0	0.71	± 0.31	44.7	± 40.6
Ad libitum-time	4.0	± 1.8	21.0	± 18.7	0.29	± 0.18	22.4	± 21.4
Food Eaten (g)								
Time-restricted	1.3	± 0.39	1.20	± 0.62	0.71	± 0.31	1.2	± 0.34
Ad libitum-time	1.2	± 0.15	1.20	± 0.71	0.29	± 0.18	1.1	± 0.66
Male Preference (time with male-time with food)/total time								
Time-restricted	0.67	± 0.15	0.53	± 0.12	0.98	± 0.01	0.60	± 0.06
Ad libitum-time	0.38	± 0.13	0.54	± 0.12	0.95	± 0.02	0.49	± 0.10

Table 2.2

	E Dose	Length of restriction 4 Days		8 Days		Length of refeeding 4 Days		8 Days	
		Duration of lordosis (sec)							
Food-restricted	1.5 µg	310	± 128	156	± 108	158	± 102	339	± 109
	2.5 µg	301	± 107	0	± 0	126	± 90	151	± 89
	20.0 µg	364	± 88	154	± 85	388	± 96	382	± 96
	Veh	0	± 0	0	± 0	0	± 0	0	± 0
Ad libitum	1.5 µg	548	± 5	345	± 105	423	± 46	411	± 73
	2.5 µg	367	± 60	340	± 60	242	± 60	401	± 43
	20.0 µg	470	± 59	428	± 63	422	± 49	511	± 27
	Veh	0	± 0	0	± 0	0	± 0	0	± 0
		Food hoarded (g)							
Food-restricted	1.5 µg	0	± 0	47	± 29	52	± 27	8	± 5
	2.5 µg	14	± 12	100	± 26	25	± 26	72	± 32
	20.0 µg	10	± 9	64	± 32	1	± 1	0	± 0
	Veh	56	± 30	69	± 28	57	± 30	25	± 24
Ad libitum	1.5 µg	2	± 1	28	± 28	30	± 30	31	± 31
	2.5 µg	1	± 1	4	± 3	6	± 3	2	± 1
	20.0 µg	0	± 0	7	± 4	32	± 24	33	± 25
	Veh	23	± 19	26	± 25	16	± 16	26	± 25
		Food eaten (g)							
Food-restricted	1.5 µg	1.6	± 0.4	1.3	± 0.5	0.7	± 0.3	0.6	± 0.2
	2.5 µg	0.5	± 0.2	0.9	± 0.6	1.0	± 0.6	0.8	± 0.5
	20.0 µg	1.0	± 0.2	1.4	± 0.4	0.7	± 0.2	0.2	± 0.1
	Veh	1.7	± 0.5	1.5	± 0.7	0.8	± 0.5	1.7	± 0.5
Ad libitum	1.5 µg	0.1	± 0.1	0.1	± 0.1	0.3	± 0.2	0.4	± 0.1
	2.5 µg	0.8	± 0.4	0.8	± 0.3	0.6	± 0.2	0.8	± 0.6
	20.0 µg	0.4	± 0.2	0.5	± 0.4	0.4	± 0.2	0.4	± 0.2
	Veh	0.7	± 0.5	0.3	± 0.2	1.5	± 0.6	1.1	± 0.6

Table 2.3

Male preference (time with male-time with food)/total time									
		Length of restriction				Length of refeeding			
	E Dose	4 Days		8 Days		4 Days		8 Days	
Food-restricted	1.5 µg	0.14	± 0.10	0.10	± 0.07	0.40	± 0.05	0.43	± 0.04
	2.5 µg	0.37	± 0.16	0.35	± 0.13	0.53	± 0.04*	0.62	± 0.02*
	20.0 µg	0.44	± 0.12	0.04	± 0.10	0.48	± 0.06*	0.53	± 0.07*
	Veh	0.55	± 0.06	0.16	± 0.15	0.64	± 0.01	0.63	± 0.02
Ad libitum	1.5 µg	0.36	± 0.09	0.22	± 0.10	0.09	± 0.13	0.15	± 0.12
	2.5 µg	0.55	± 0.05	0.51	± 0.13	0.62	± 0.02	0.59	± 0.05
	20.0 µg	0.41	± 0.12	0.37	± 0.17	0.46	± 0.09	0.47	± 0.06
	Veh	0.55	± 0.05	0.60	± 0.02	0.57	± 0.04	0.63	± 0.01

Table 2.4

Time After Implant	6 Hours	12 Hours	30 Hours	54 Hours	150 Hours
Time After Injection				6 Hours	6 Hours
Length of Food Restriction	4 Days	4 Days	5 Days	6 Days	10 Days
Number of Vaginal Marks					
Estradiol	1.60 ± 0.61	1.00 ± 0.48	2.87 ± 1.13*	0.71 ± 0.42*	0.29 ± 0.18
E+P	-	-	-	0 ± 0	0.13 ± 0.13
Progesterone	-	-	-	0 ± 0	0 ± 0
Vehicle	0.53 ± 0.47	0.67 ± 0.49	0 ± 0	0 ± 0	0 ± 0
Number of Flank Marks					
Estradiol	0.60 ± 1.98	2.27 ± 1.12	3.73 ± 1.61*	1.86 ± 1.24	0.57 ± 0.43
E+P	-	-	-	0 ± 0	0.13 ± 0.13
Progesterone	-	-	-	1.50 ± 1.23	0 ± 0
Vehicle	1.67 ± 0.97	0.73 ± 0.46	0.93 ± 0.44	0.14 ± 0.14	0.29 ± 0.18

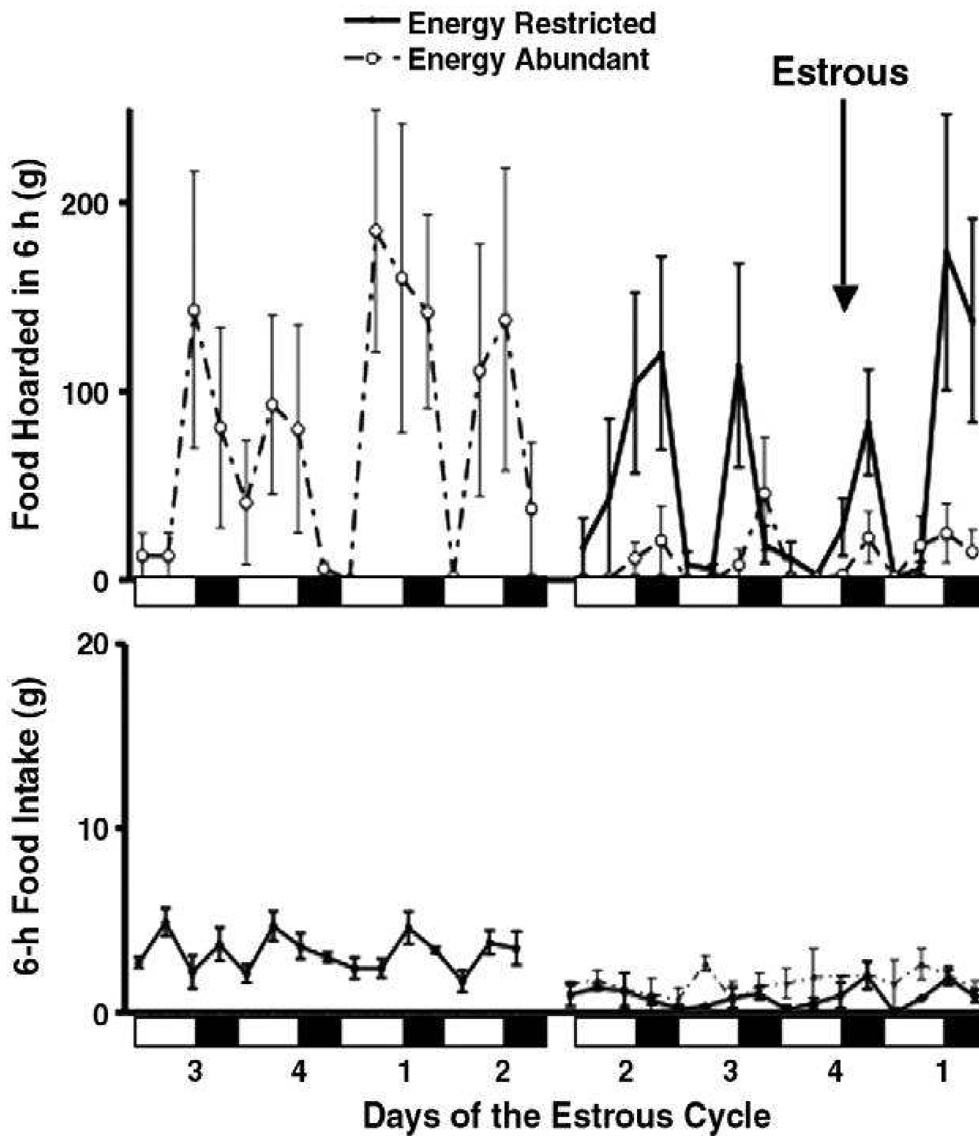
Table 2.5

Time After Implant	6 Hours	12 Hours	30 Hours	54 Hours	150 Hours
Time After Injection				6 Hours	6 Hours
Length of Food Restriction	4 Days	4 Days	5 Days	6 Days	10 Days
Food Eaten in 90 min (g) with Male and Female Stimuli					
Estradiol	0.36 ± 0.10	0.67 ± 0.15	0.92 ± 0.26	0.65 ± 0.28	0.11 ± 0.07
E+P	-	-	-	0.48 ± 0.23	0.09 ± 0.08
Progesterone	-	-	-	0.95 ± 0.49	0.35 ± 0.33
Vehicle	0.55 ± 0.18	0.86 ± 0.30	0.94 ± 0.28	0.96 ± 0.30	0.55 ± 0.23
Food Eaten in 90 min (g) in Absence of Male and Female Stimuli					
Estradiol	-	-	-	0.36 ± 0.17	0.76 ± 0.42
E+P	-	-	-	0.32 ± 0.14	0.97 ± 0.48
Progesterone	-	-	-	0.65 ± 0.41	0.40 ± 0.38
Vehicle	-	-	-	0.25 ± 0.15	0.26 ± 0.21

Table 2.6

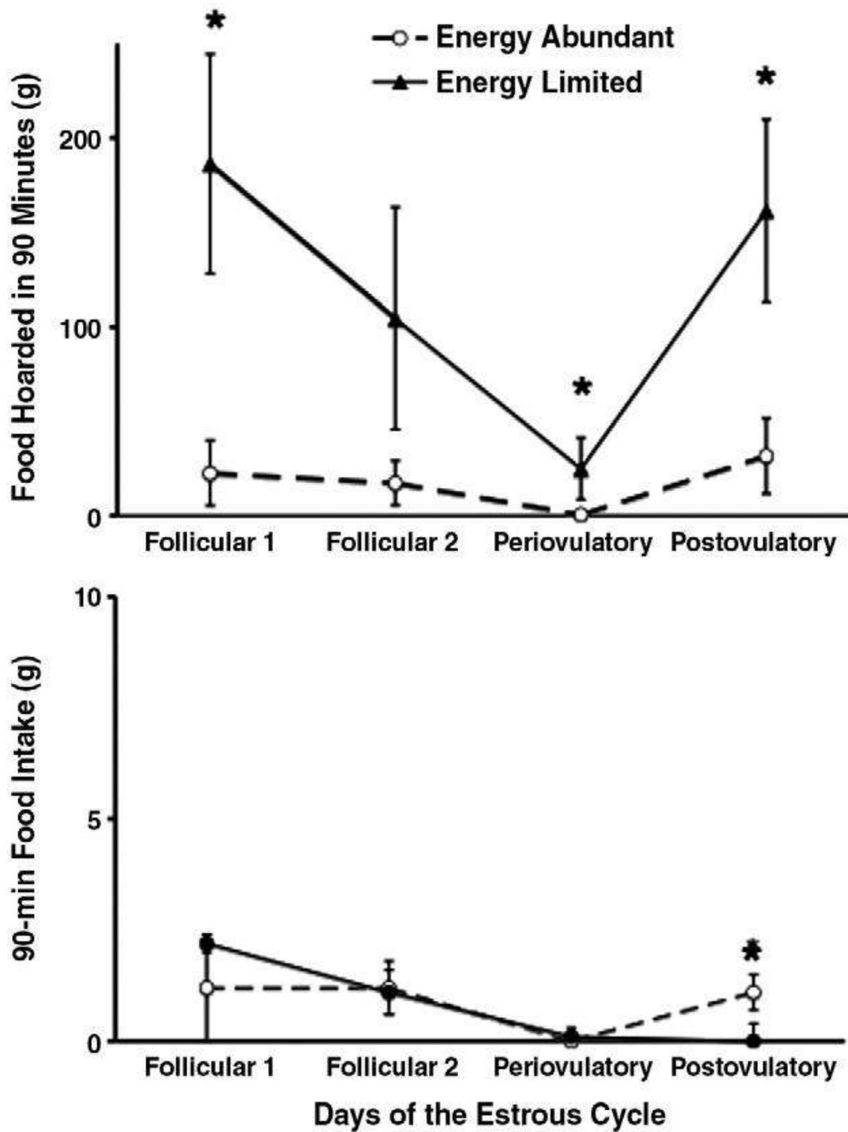
	Estradiol			E+P			Progesterone			Vehicle		
10-d Δ body weight	-18.82	±	2.76	-15.41	±	2.39	-17.37	±	3.44	-17.47	±	2.04
Visceral (total weight in g)	1.28	±	0.32	1.28	±	0.41	1.57	±	0.37	1.53	±	0.36
Intraperitoneal (total weight in g)	0.38	±	0.05	0.39	±	0.07	0.55	±	0.15	0.49	±	0.09
Subcutaneous (unilateral weight in g)	0.82	±	0.16	0.81	±	0.18	1.11	±	0.18	0.97	±	0.16
Gonadal (unilateral weight in g)	0.37	±	0.10	0.25	±	0.05	0.44	±	0.14	0.35	±	0.11
Perirenal (unilateral weight in g)	0.20	±	0.04	0.26	±	0.05	0.20	±	0.03	0.23	±	0.05

Figure 2.1



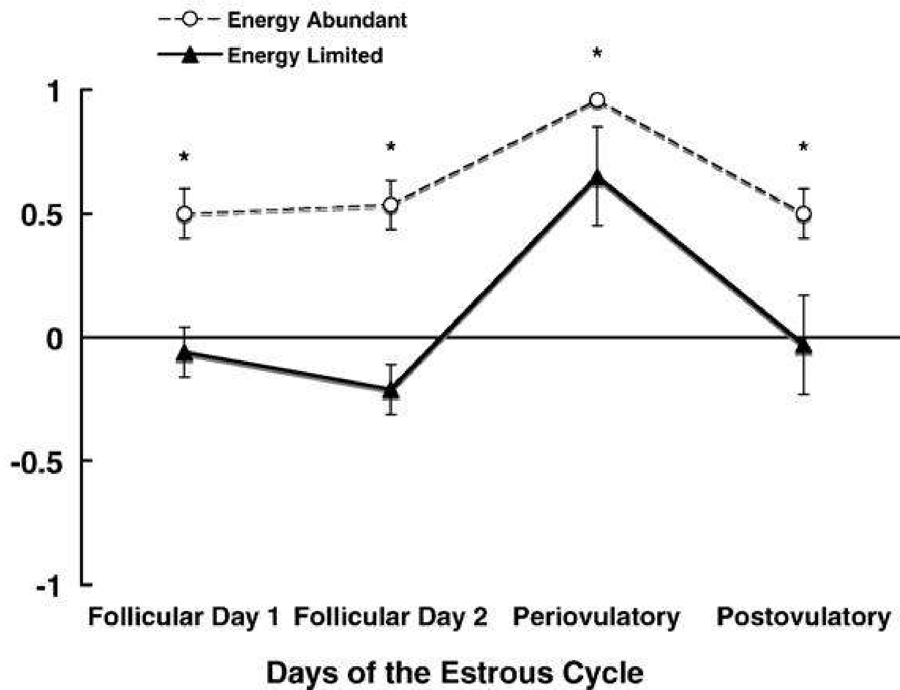
Mean and standard error of the mean for amount of food hoarded (top) and food intake (bottom) in hamsters tested every 6 h of the 4-day estrous cycle. All females were singly housed. All were fed ad libitum prior to and during testing for the first 6 days (hatched lines, open circles), and half food-restricted (solid lines, filled circles), half fed ad libitum for the last 6 days of testing.

Figure 2.2



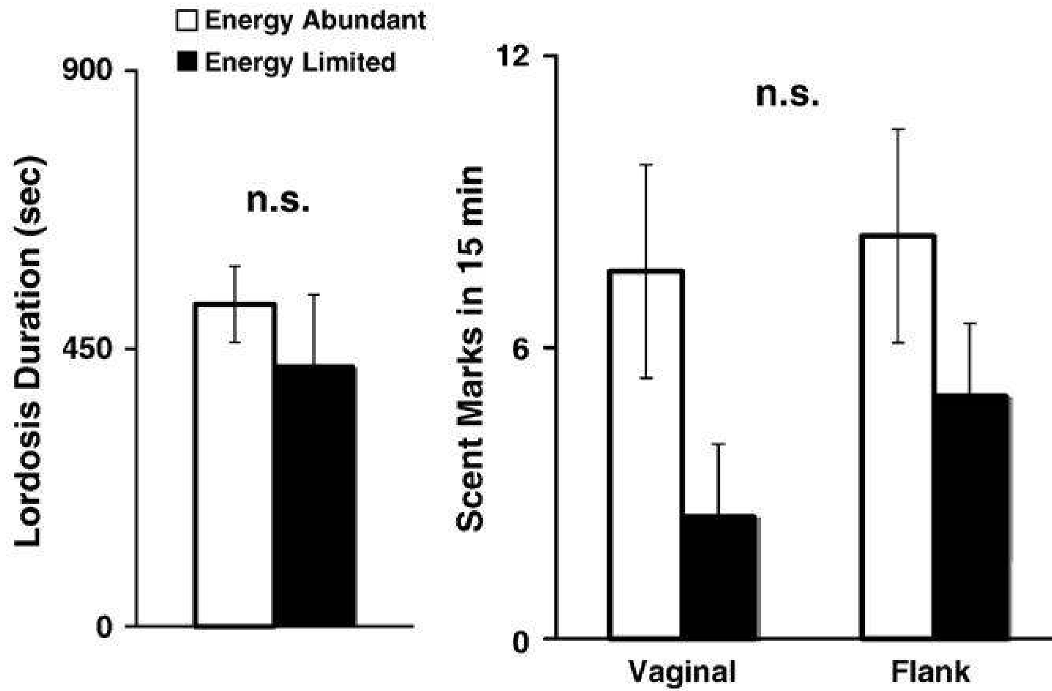
Mean and standard error of the mean for the amount of food hoarded (top) and food intake (bottom) in hamsters tested during a 90 min period that spanned the onset of the dark phase of the photoperiod each day of the 4-day estrous cycle. Female hamsters were either fed ad libitum ("Energy-abundant") or food-restricted to 75% of their baseline ad libitum daily intake ("Energy-limited") for 8 days before testing. * Significantly different from ad libitum at $P < 0.05$.

Figure 2.3



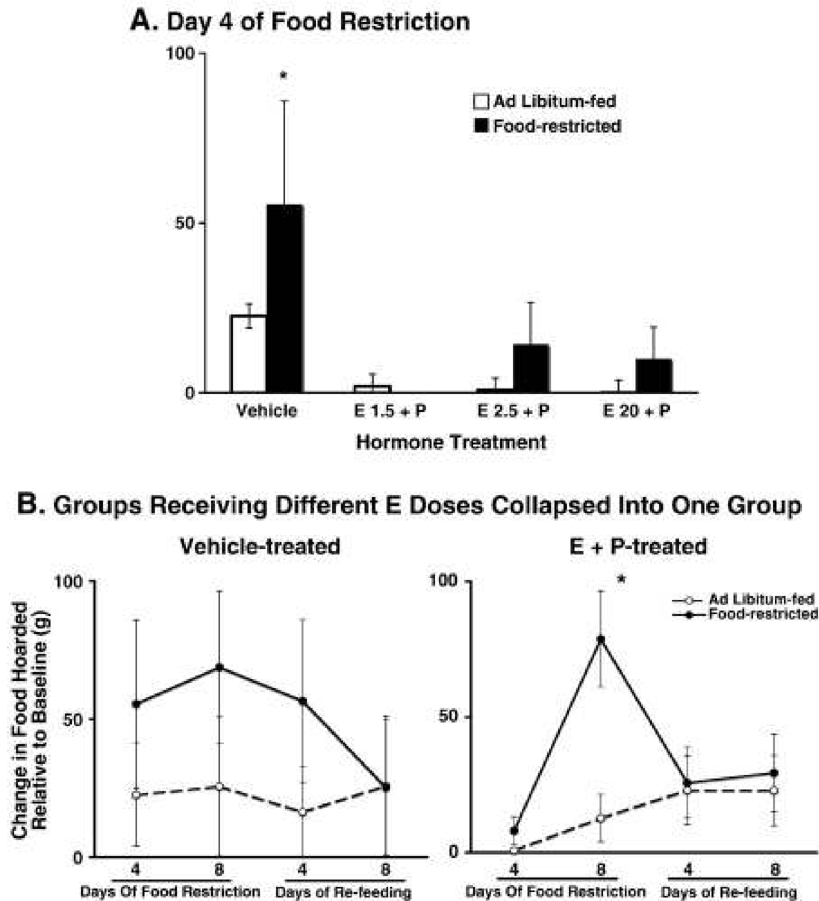
Mean and standard error of the mean for male preference, calculated as the (time spent with males – the time spent with food) / the total time. Preference was measured during a 15 min test each day of the 4-day estrous cycle. Female hamsters were either fed ad libitum ("Energy-abundant") or food-restricted to 75% of their baseline ad libitum daily intake ("Energy-limited") for 8 days before testing. * Significantly different from ad libitum at $P < 0.05$.

Figure 2.4



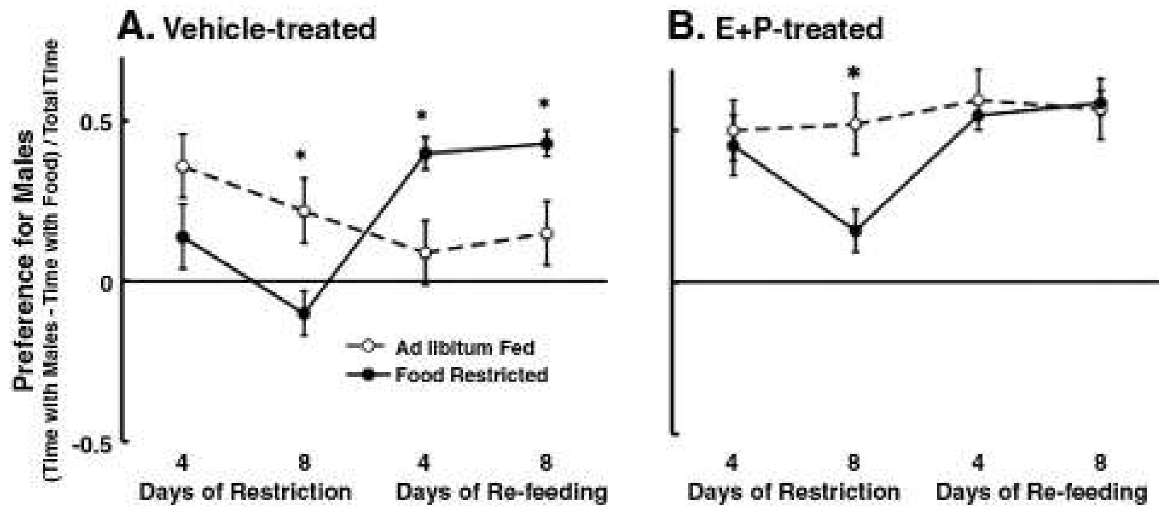
Mean and standard error of the mean for lordosis duration (left), and scent marking, both vaginal and flank (right) in female Syrian hamsters. Female hamsters were either fed ad libitum ("Energy-abundant") or food-restricted to 75% of their baseline ad libitum daily intake ("Energy-limited) for 8 days before testing. n.s. = Not significantly different by $P < 0.05$.

Figure 2.5



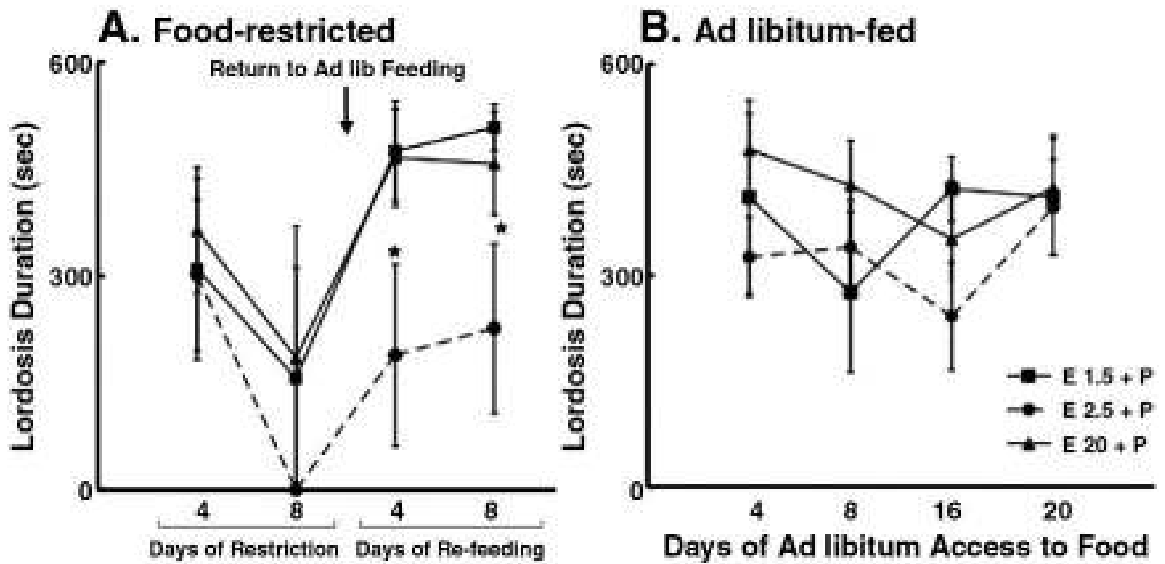
A) Mean and standard error of the mean for amount of food hoarded in hamsters tested during a 90 min period near the onset of the dark phase of the photoperiod. Hamsters were treated by s.c. injection with 500 µg progesterone or the oil vehicle 6 h before testing and with different doses of estradiol (1.5, 2.5, or 20 µg) or the oil vehicle 48 h before testing. Half of each group was either fed ad libitum or food-restricted to 75% of their baseline ad libitum daily intake. B) Mean and standard error of the mean for amount of food hoarded in hamsters treated with vehicle (left) or estradiol plus progesterone (right). Females from three dose groups collapsed into one group. * Significantly different from ad libitum at $P < 0.01$.

Figure 2.6



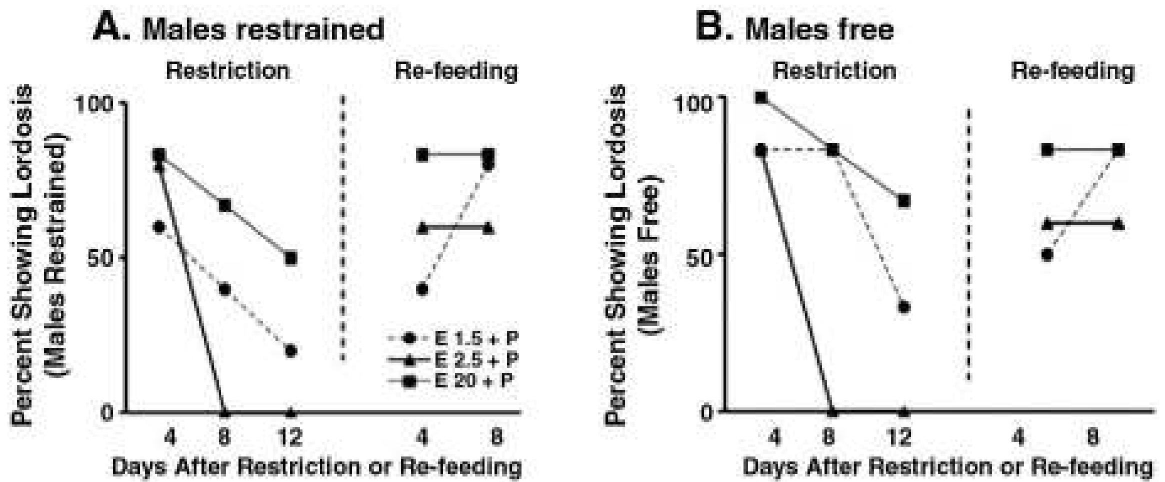
Mean and standard error of the mean for male preference in hamsters A) tested at 4 and 8 days of food restriction or ad libitum feeding, and B) after 4 and 8 days of return to ad libitum feeding. Preference was calculated as the (time spent with males – the time spent with food) / the total time. Hamsters were treated by s.c. injection with 500 µg progesterone 6 h before testing and with different doses of estradiol (1.5, 2.5, 20 µg) or the oil vehicle 48 h before testing. * Significantly different form ad libitum at $P < 0.05$.

Figure 2.7



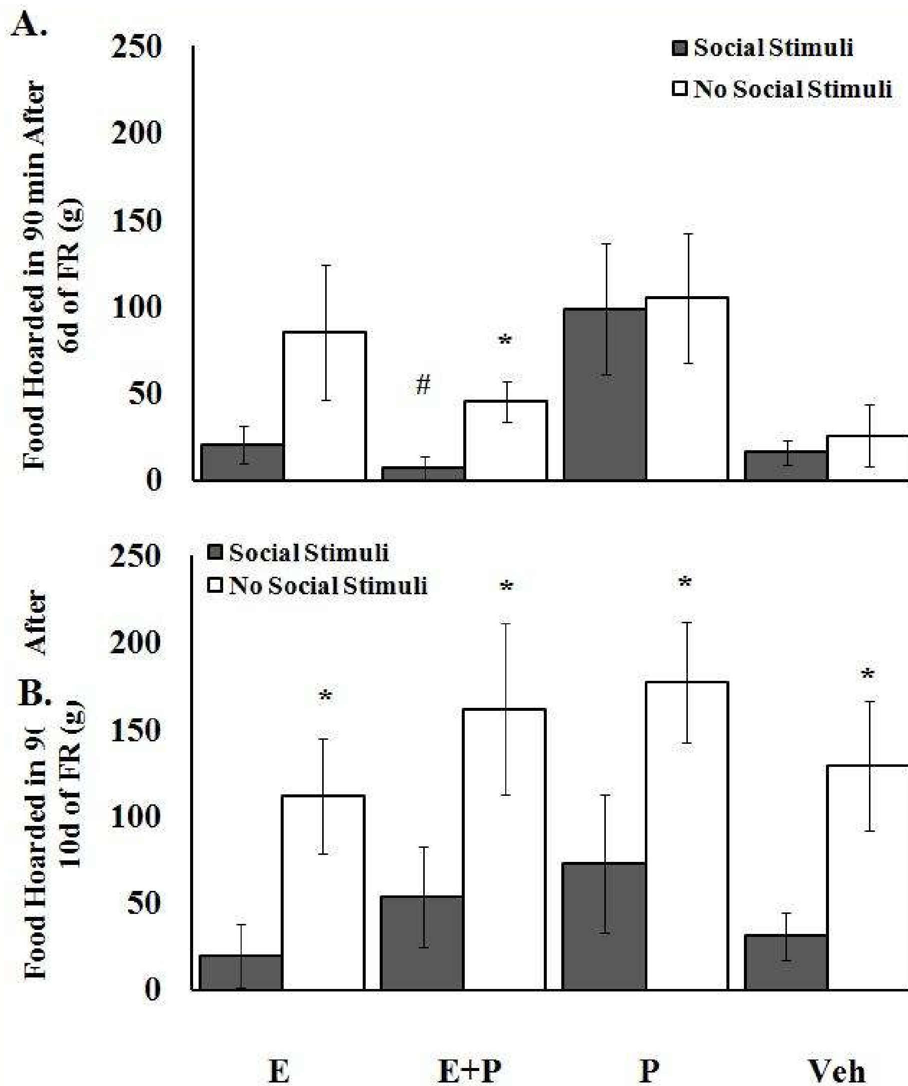
Mean plus standard error of the mean for lordosis duration in females either A) restricted to 75% of their baseline daily food intake for 4, 8, or 12 days and then returned to ad libitum food access for 4 and 8 days, or B) fed ad libitum during the entire experiment. All females were ovariectomized and treated with 500 µg progesterone 6 h before testing and one of three doses of estradiol benzoate: 1.5, 2.5, or 20 µg 48 h before testing. Vehicle-treated hamsters did not show lordosis and data are not shown. * Significantly different from other two estradiol dose groups at $P < 0.05$.

Figure 2.8



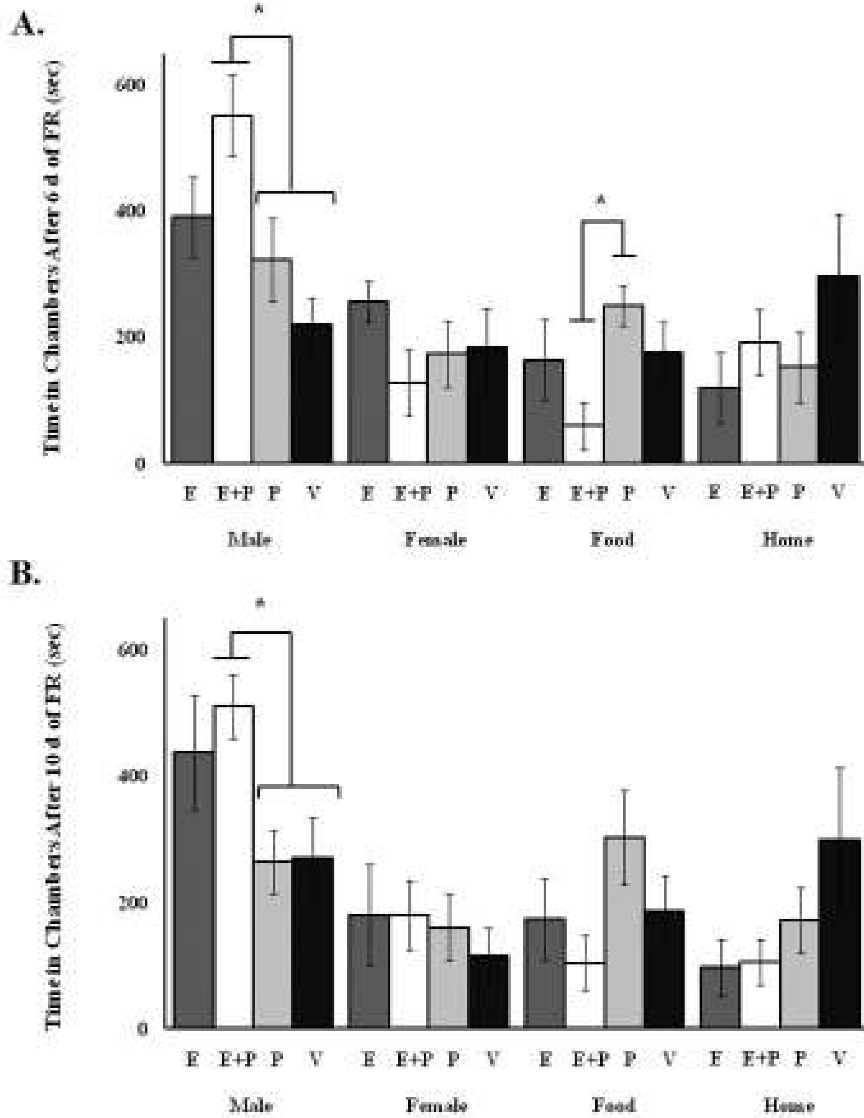
Percent of food-restricted hamsters showing lordosis when tested with A) restrained males or B) with males restrained behind a wire cage. In the former group, females received gentle flank stimulation by the experimenter. All females were ovariectomized and treated with 500 µg progesterone 6 h before testing and one of three doses of estradiol benzoate: 1.5, 2.5, or 20 µg 48 h before testing. Vehicle-treated hamsters did not show lordosis and data are not shown.

Figure 2.9



Mean and standard error of the mean for 90-min food hoarding after A) 6 days of food restriction or B) 10 days of food restriction in the presence or absence of social stimuli. All females were OVX and food-restricted (fed 75% of their ad libitum intake) for 4 days prior to implantation. 48 and 144 h after the capsules were implanted, females were given a S.C. injection of progesterone or vehicle (canola oil) and tested in the preference apparatus. * = Significantly greater than all other groups at P < 0.05.

Figure 2.10



Mean and standard error of the mean for the time spent in each chamber of the apparatus after A) 6 days of food restriction or B) 10 days of food restriction. All females were OVX and food-restricted (fed 75% of their ad libitum intake) for 4 days prior to implantation. 48 and 144 h after the capsules were implanted, females were given a S.C. injection of progesterone or vehicle (canola oil) and tested in the preference apparatus.
* = Significantly different at $P < 0.05$.

Chapter 3

Food Restriction Dissociates Sexual Motivation, Sexual Performance, and the Rewarding Consequences of Copulation in Female Syrian Hamsters

Hormonal control of ingestive behavior is relevant to clinical problems such as obesity, metabolic syndrome, infertility, and addiction. An understanding of the neuroendocrine mechanisms underlying ingestion requires attention to the functional significance of hormone action for both survival and reproductive success (Klingerman et al., 2010; Schneider, 2006; Schneider et al., 2007). For example, many investigators assume that the neuroendocrine mechanisms underlying ingestive behavior function to maintain body weight within narrow limits. Mounting evidence, however, suggests that many of these mechanisms function to prioritize conflicting behaviors in order to optimize reproductive success in environments where energy fluctuates (Klingerman et al., 2010; Schneider, 2006; Schneider et al., 2007). Our experimental approach has been to examine the effects of food availability on the choice between food and sexual stimuli made by subjects housed and tested in semi-natural environments. The advantage of this approach lies in the fact that many neural mechanisms exist, not only because they maintain a particular body size, but also (or primarily) because they conferred reproductive success during evolutionary history.

The present experiments are focused on the neural mechanisms that mediate female motivation and reward underlying the choice between food and a mating partner

in Syrian hamsters (*Mesocricetus auratus*). Hamsters exhibit regular, 4-day estrous cycles and easily-quantifiable sex and ingestive behaviors, and can be housed in an apparatus that mimics important aspects of the natural habitat. Syrian hamsters in the wild live in isolation in underground burrows, from which they emerge for 80 to 90 min per day, primarily for foraging and hoarding (Gattermann et al., 2008). Mating has been observed in the wild, and was reported to occur just outside the female's burrow (Gattermann et al., 2008). Thus, in the present experiments, estrous-cycling females were housed in home cages, but were free to forage for 90 min per day via vertical tunnels leading in one direction to food and in another direction to an adult male.

Motivated behaviors such as eating and copulating have at least three components: motivation, performance and reward. Consummatory behaviors might, in some cases, reflect both motivation and performance. Some appetitive sex behaviors, however, are at least partially distinct from consummatory sex behaviors. Appetitive sex behaviors occur separated in time from copulation, reflect sexual motivation but not necessarily the ability to perform the sex act, serve to bring animals in close contact with opposite-sex conspecifics, and are assumed to induce arousal in the potential mating partner (Craig, 1917; Everitt, 1990; Johnston, 1974; Johnston, 1977; Lisk, 1983; Lorenz, 1950; Sherrington, 1906). For example, Syrian hamsters respond to male odors by making vaginal scent marks and by spending more time in closer proximity to males on day 3 of the estrous cycle, one full day before ovulation and the consummatory act of mating (Johnston, 1974; Johnston, 1975). Syrian hamster appetitive ingestive behaviors include food hoarding, the latency to eat food, and the preference for food over sex. In

contrast to appetitive behaviors, consummatory behaviors include food intake (the amount of food eaten within a particular time period) and the percentage of females that show the lordosis reflex on day 4 of the estrous cycle in an enclosed area in response to an adult, sexually-experienced male. Sex and other behaviors are said to have rewarding consequences when performance of these behaviors increases their future occurrence in the contexts in which they first occurred. Sexual reward in hamsters has been measured using the conditioned place preference (CPP) method, in which an estrous female develops a preference for a chamber previously associated with mating (Meisel et al., 1996).

The extent to which appetitive, consummatory, and reward mechanisms are distinct is unknown. Experiment 3.1 was designed to determine whether there was a level of food restriction that could inhibit appetitive sex and ingestive behaviors without effects on consummatory behaviors. Experiments 3.2 and 3.3 were designed to determine whether the level of food restriction that inhibited appetitive sex behaviors also inhibited the rewarding consequences of copulation both in terms of formation of a CPP and neural activation of brain areas thought to mediate reward.

Materials and Methods

Animals and Housing

The subjects were adult (at least 3 months of age) female Syrian hamsters obtained from the breeding colony at Lehigh University (original stock from Charles River Laboratories) or directly from Charles River Laboratories (Stoneridge, NY).

Hamsters were used at 140.0 ± 10 g body weight in Experiment 3.1, 148.0 ± 10 g in Experiment 3.2, and 148.0 ± 16 g in Experiment 3.3. Hamsters were housed individually in plastic laboratory cages (either 50 × 40 × 10 cm or 31 × 19 × 18 cm) with wire lids. The colony environment was maintained at 22°C with a 14:10 light-dark cycle (lights on at 2200 h) and food and water were provided ad libitum until the point at which half of the hamsters were food-restricted to 75% of the 4-day average of their ad libitum daily food intake. All procedures followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, the United States Department of Agriculture, and a protocol approved by the University of Minnesota and Lehigh University Institutional Animal Care and Use Committees.

Estrous Cyclicity

Ovulation and estrous behavior occur on the last day of the 4-day estrous cycle in Syrian hamsters. Increased circulating concentrations of estradiol on days 3 and 4 culminates in the luteinizing hormone surge and ovulation. Estradiol and progesterone are critical for estrous behavior (the stationary, arched-back lordosis posture necessary for the male to gain intromission). Estrous cycle day 3 is characterized by an increase in circulating estradiol, a decrease in aggression, an increased tolerance for males, and increased vaginal scent marking, but no lordosis.

Only females that showed two consecutive 4-day estrous cycles were chosen for each experiment. Estrous cyclicity was determined by brief introduction of an adult male stimulus hamster into the female's cage. Daily tests for estrous behavior (lordosis) were

carried out each day within 30 min of the onset of the dark phase of the light-dark cycle until 4-day estrous cycles were established. Females were considered to be in heat (day 4 of the estrous cycle) if they showed lordosis within 5 min. Those that did not display lordosis within this time period were typically aggressive toward the male and the test was terminated; testing was resumed at the same time the next day. Tests for other behaviors (vaginal scent marking, male preference, food hoarding, etc.) occurred throughout the 20 days of food-restriction or ad libitum-feeding, even if the females failed to show lordosis on the expected day of estrous.

Food Restriction

Individually-housed females were given a pre-weighed amount of food (approximately 20 g) that was measured to the nearest 0.01 g each day at 1200 hours for 4 days. The baseline daily ad libitum food intake was calculated as the difference in the weight of food found in the cage each subsequent day. Mean daily ad libitum food intake (the sum of 4 daily food intakes divided by 4) was determined for each female. Food-restricted females received 75% of their ad libitum food intake divided into two rations given 12 h apart. Females were tested 12 h after their last food ration had been given, and no food was found in the cage at the time of testing.

Procedures

Experiment 3.1. Time Course of Effects of Food Restriction on Appetitive and Consummatory Behaviors

To test the idea that deficits in energy availability set behavioral priorities at the level of motivation rather than performance, we examined the time course of changes in sex and ingestive behaviors in estrous-cycling female Syrian hamsters every 4 days for 20 days of mild (25%) food restriction and after return to ad libitum food intake. This allowed us to pinpoint the duration of food restriction that significantly affected motivation but not performance, and to look for correlated changes in the mesolimbic dopamine system and other brain areas in Experiment 3.2, and changes in the formation of a CPP to copulation in Experiment 3.3.

The Preference Apparatus

In order to examine the motivation to engage in sex or ingestive behaviors, 16 hamsters (weights and ages described in Animals and Housing) were introduced to a preference apparatus described previously (Klingerman et al., 2010). Each apparatus contained a home cage connected via plastic tubes to a food source box and another box that contained an adult male. The home cage was made from an opaque, Nalgene cage (31 × 19 × 18 cm) lined with fine wood shavings with a door that was kept closed when the animal was not being trained or tested. When the subject females were being trained or tested, the door to the home cage was opened, which allowed the females to ascend a vertical tube (134 cm in length) leading to 2 horizontal tubes (40-50 cm in length), connected in a T-configuration. One horizontal tube was connected to a clear, plastic box (the food box), containing a weighed amount (150 ± 5 g) of hoardable pellets. Hoardable pellets were made from standard laboratory chow (Harlan Rodent Chow 2016) that was

broken into 2 cm pieces, a size that hamsters can easily fit into their cheek pouches and carry through the plastic tubes. A second horizontal tube was connected to a clear, Plexiglas cage (27 × 20 × 15 cm), containing an adult, sexually-experienced male (the male box). During assessment of appetitive behaviors, the male was separated from the female by a wire barrier that allowed olfactory, visual and tactile stimuli from the male, but prevented the male from mating with the female. The male box did not contain food or water.

Subject females were acclimated to the home cage that contained fine wood chip bedding, food and water for at least 1 week prior to testing. This reduced any tendencies to sleep or move bedding to other chambers.

Training to the Preference Apparatus

Once subjects were acclimated to the home cage, they were trained to expect food in the food box and a male in the male box for four consecutive days. Subjects were trained to each box separately, by blocking access to opposing tubes for 90 min. On days 1 and 2 of the estrous cycle, females were trained to the food box and allowed to keep the food they hoarded in their home cages. On days 3 and 4, females received additional training with the male box. On the eve of day 3, females were allowed to enter into the male box where an unrestrained, gonadally-intact male was housed (females cannot become impregnated on this day). Females were allowed to interact with the males for 5 min or until a fight broke out. On the eve of day 4, the day of estrous, females were allowed to enter the male box, to interact with the male, and receive anogenital licks and

ectopic mounts without intromissions or ejaculations for 5 min. During training, the experimenter prevented male hamsters' intromission so that no females would become pregnant.

Testing in the Preference Apparatus

Testing began at the onset of the dark phase of the light-dark cycle (1200 h) and was conducted under dim, red illumination on days 3 and 4 of the estrous cycle. To start the test, the door to the home cage was opened and females were allowed access to both the food and male boxes for a total of 90 min. The first 15 min of the 90 min test were conducted while the experimenter recorded all behaviors every 5 sec. In the remaining 75 min of the test, the females were allowed to move freely about the apparatus, interact with males, hoard or eat food, or remain in the home cage. During this period the experiment did not record the subjects' behaviors. Behaviors that were recorded during the 15 min on day 3 included vaginal marking, flank marking, hoarding, and eating. At the end of the full 90 min, all food was weighed in the food box and the home cage. No food was found in the male box.

On day 4 of the estrous cycle, females received a 10 min test with a gonadally-intact, male hamster restrained behind a wire barrier in the male box. After the female hamster entered the male box, stimulation was provided to her flanks by an experimenter to induce the lordosis reflex (while the male continued to be restrained behind the wire barrier). The latency and duration of the females' lordosis was recorded.

Previous research showed that food-restricted females that do not show lordosis when the male is restrained will often show lordosis when the male is allowed to mount (Klingerman et al., 2010). Thus, in the present experiment, if the females did not show lordosis during the 10 min period, the wire barrier was removed and the male hamster was allowed to mount the female, and the occurrence or absence of lordosis was recorded. The male was quickly removed if a fight broke out between the two hamsters.

Following the lordosis test with the free male, recording was stopped and the male was placed back behind the wire barrier and the test continued for an additional 80 min while the female still had access to both the food box and the male box. Throughout the total 90 min, the females were free to hoard food, eat food, investigate the male, travel through the tube, or remain in the home cage. At the end of the full 90 min, all food was weighed in the food box and the home cage.

Food hoarding was measured as the difference between the weight of food in the food box at the start of the 90 min and the weight of the food left in the food box at the end of the 90 min. Food intake was measured as the sum of the food in all compartments (home + food box) at the start of 90 min minus the sum of the food in all compartments at the end of 90 min.

Baseline behaviors were measured on days 3 and 4 of the estrous cycle prior to the start of food restriction. Females were randomly placed into one of two groups that did not differ significantly in body weight. During 20 days of food restriction or ad libitum feeding, all hamsters were tested in the preference apparatus on days 3 and 4 of each subsequent estrous cycle. Thus, they were tested on days 3 and 4, 7 and 8, 11 and

12, 15 and 16, and 19 and 20 of restriction and days 3 and 4, and 7 and 8 after return to ad libitum feeding.

After 16 days of restriction when the majority of food-restricted hamsters stopped showing lordosis, those that failed to show lordosis were returned to ad libitum feeding, whereas the rest were food-restricted for 1 more cycle. After 20 days of restriction, the remaining hamsters were returned to ad libitum feeding whether they showed lordosis or not. After the return to ad libitum feeding, hamsters were re-tested in the preference apparatus on days 3 and 4 for two additional estrous cycles.

Statistical Analysis

Data were analyzed using repeated measures analysis of variance (ANOVA) to determine whether there were changes over time, and whether these effects differed according to food-restriction or ad libitum feeding. Repeated measures ANOVA were performed on the amount of food hoarded and eaten during the 90-min period, male preference ((the time with the male minus the time with the food)/total time), and the number of vaginal and flank marks across all time points of restriction followed by planned contrasts when the main effects were significant. The percentages of hamsters displaying lordosis were compared using Fisher's exact probability test. Differences were considered significant at $P < 0.05$.

Experiment 3.2. Mating-induced Neural Activation in the Nucleus Accumbens (NAc) in Food-restricted and Ad libitum-fed Females

This experiment was designed to determine whether food restriction could dissociate appetitive behaviors from the neural activation known to occur as a consequence of copulatory experience. In Experiment 3.1, appetitive sex behaviors were not inhibited by 7 days of food restriction, but were significantly inhibited by 11 days of food restriction at 75% of ad libitum intake (Fig. 3.1A), with no significant effect on consummatory behavior in the majority (4/6) of females (Fig. 3.1B). Because Experiment 3.2 required females in which appetitive, but not consummatory behaviors were inhibited, we used 10 days of food restriction in Experiment 3.2. We knew 7 days would be unlikely to inhibit appetitive behavior, and that 8 days was sufficient to inhibit appetitive sex behavior in a previous experiment (Klingerman et al., 2010), but 12 days might be to long, since there were 2 females in Experiment 3.1 that failed to show lordosis after 12 days of restriction.

The mesolimbic dopamine system, including the nucleus accumbens (NAc), is of interest because this brain area is thought to play a role in appetitive behavior, (Alcaro et al., 2007; Bassareo and Di Chiara, 1999; Ikemoto and Panksepp, 1999; Mitchell and Gratton, 1994; Salamone, 1994), effortful approach to reinforcing stimuli (Salamone et al., 2009), and is activated by experience with addictive drugs (Di Chiara and Imperato, 1988; Nestler, 2001) and naturally rewarding stimuli including copulatory experience (Kalivas and Volkow, 2005; Kohlert and Meisel, 1999; Lajtha and Sershen, 2010; Meisel and Joppa, 1994; Salamone et al., 2005). Whether there is increased release of dopamine and neural activation after vaginal scent marking or other appetitive behaviors in hamsters is unknown, and since mild food restriction decreases these behaviors, it would

be difficult to determine whether food restriction also inhibits the rewarding aspects of these behaviors. However, there are instances where the rewarding aspects of copulation are inhibited even when the performance of copulation is not inhibited. In Syrian hamsters, for example, copulatory experience increases neural activation and dopamine release in the NAc (Bradley and Meisel, 2001; Kohlert and Meisel, 1999), and antagonists to dopamine receptors block the rewarding aspects of sexual experience without decreasing copulatory behavior (lordosis) (Meisel et al., 1996). Perhaps food restriction, like a dopamine receptor antagonist, also decreases the rewarding consequences of copulation. In Experiment 3.2, we compared food-restricted to ad libitum-fed females' mating-induced neural activation in the NAc, as measured by immunoreactivity to c-Fos, the protein product of the immediate-early gene, c-fos. If brain mechanisms involved in reward, specifically the NAc, share neuroanatomical and functional overlap with mechanisms that control appetitive behavior, it would be expected that food restriction would attenuate mating-induced neural activation in the NAc. However, no attenuation of neural activation would be expected in brain areas more strictly involved in lordosis, such as the ventromedial hypothalamus (VMH) or medial nucleus of the amygdala (MeA). Alternatively, mating-induced neural activation in the NAc might be dissociated from appetitive behaviors by mild food restriction.

c-Fos immunoreactive cells were also counted in brain areas containing various peptides implicated in energetic control of food intake and reproduction, such as the arcuate nucleus (Arc) and paraventricular nucleus of the hypothalamus (PVN), which contain leptin receptors, neuropeptide Y/agouti-related protein, proopiomelanocortin and

corticotropin releasing hormone (Backholer et al., 2009; Baldo et al., 2003; Bentley et al., 2006; Hakansson et al., 1998; Jones et al., 2004; Mercer et al., 1998; Qi et al., 2009; Seymour et al., 2005; Tartaglia et al., 1995).

Animal Testing

Twenty-four female hamsters were either fed ad libitum or food-restricted for 10 days as described under Food Restriction (weights and ages reported above in Animals and Housing). On the tenth day, half of the females from each group in Experiment 3.2 (6 food-restricted hamsters; 6 ad libitum-fed hamsters) received a 10 min mating test in which a sexually naïve male stimulus hamster was introduced to each female's cage and mating behavior was video recorded. All tests were conducted within the first 2 h of the dark portion of the light-dark cycle. The mating behavior recorded included lordosis latency, total lordosis duration, and total numbers of mounts, intromissions, and ejaculations. Hit rate (proportion of mounts that included intromission) was derived, and for this purpose, ejaculations were scored as intromissions.

Fos Immunocytochemistry

One h following sex testing, females were deeply anesthetized with Sleepaway (Nembutal, Fort Dodge Laboratories, Fort Dodge, IA 0.2 ml/animal, I.P.) and intracardially perfused with 25 mM phosphate-buffered saline (PBS, pH 7.6, 4°C) for 2 min followed by 4% paraformaldehyde in 25 mM PBS (4°C) for 20 min. Brains were post-fixed in 4% paraformaldehyde for 2 h and cryoprotected in 10% sucrose in PBS at

4°C overnight. Coronal 40 µm sections were cut on a freezing microtome at the level of the NAc, and at the level of the MeA/VMH and Arc. Sections were rinsed in wash buffer (25 mM PBS + 0.1% BSA) and incubated in c-Fos primary antibody (Santa Cruz Biotechnologies, Inc, Santa Cruz, CA; cat. # sc 52; 1:3000 in wash buffer + 0.3% Triton X-100) for 24 h at room temperature followed by 24 h at 4°C. Following appropriate buffer washes, the sections were incubated for 45 min each in biotinylated anti-rabbit IgG (1:200 in wash buffer, Elite Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA) and avidin–biotin horseradish peroxidase complex (1:50 in wash buffer, Elite Vectastain ABC Kit, Vector Laboratories), separated by appropriate rinses in wash buffer. Sections were then further rinsed in 0.05 M Tris buffer (pH 7.6), and then incubated for 5 min in 0.07% diaminobenzidine (DAB) (Sigma-Aldrich Chemicals, St. Louis, MO) in Tris activated by 0.003% hydrogen peroxide. To intensify staining 0.015% nickel ammonium sulfate was added to the DAB reactions. Finally, the sections were rinsed with Tris buffer and deionized water and mounted on slides to dry overnight. The sections were then cleared and cover-slipped with DPX (Sigma-Aldrich Chemicals).

Slides were analyzed using a Leica DM4000B light microscope coupled to a Leica DFC500 digital camera. Brain regions analyzed included the dorsal and ventral subdivisions of the posterior dorsal MeA, the lateral and medial divisions of the ventromedial hypothalamus (VMH shell and VMH core), the NAc core and shell, the PVN, and the arcuate nucleus of the hypothalamus.

The sampling procedure insured that the counting regions were consistent among animals and that the individual who counted stained cells was blind to the experimental

condition. Counting boxes were sized to encompass most of the identified region by positioning boxes on appropriate archived digital cresyl violet stained hamster brain images previously generated in the laboratory. These counting boxes were then digitally layered onto images of immunocytochemically-processed tissue with Adobe Photoshop. Boxed images were then analyzed for number of c-Fos-IR labeled cells by using ImageJ, and were only included in the cell count if completely enclosed within the counting box. Differences among the groups were analyzed using a 2 x 2 (food restriction x test) ANOVA, followed by Student–Newman–Keuls post hoc test if the main effects were significant. Differences were considered statistically significant if P was less than 0.05.

Experiment 3.3. Effects of Energy Restriction on Formation of a Conditioned Place Preference to Mating

This experiment was designed to determine whether food restriction can dissociate appetitive sex behaviors from the formation of a conditioned place preference (CPP), a change in behavior that indicates that a stimulus has been rewarding or reinforcing.

24 ovariectomized (OVX) Syrian hamsters (weights and ages reported above in Animals and Housing) were randomly placed into treatment groups, ad libitum or food-restricted, that did not differ in body weights. Hamsters in both groups were fed 1 daily ration 4 h after the onset of the dark phase of the light-dark cycle. Females in the food-restricted group received 75% of their ad libitum food intake for 8 days until the start of conditioning in the CPP apparatus, and then their body weights were maintained for the

rest of the experiment to approximately 10 g below the body weights of the ad libitum-fed controls. After the first conditioning trial, we maintained the food-restricted females' body weights and level of restriction by alternating days of ad libitum feeding with days of restriction. Ad libitum feeding days occurred after each CPP conditioning trial, whereas restriction days occurred on the 3 days prior to the CPP conditioning trial.

Ovariectomy

Hamsters were deeply anesthetized using 80 mg/kg of sodium pentobarbital (Ovation Pharmaceuticals, Inc., Deerfield, IL) and OVX through bilateral flank incisions closed with suture (muscle incision) and wound clips (skin incision). An analgesic (Metacam, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO; 0.06 ml) was given at the time of anesthesia to minimize pain and discomfort after surgery. After at least one week of recovery, hamsters were randomly placed into treatment groups that did not differ in body weight.

Females were primed with an estrous-inducing regimen of estradiol and progesterone prior to each conditioning or testing session. Forty-eight hours before the conditioning or testing in the CPP apparatus, OVX females were given a subcutaneous (S.C.) injection of 10 µg estradiol, and six hours before testing or conditioning a S.C. injection of 500 µg of progesterone. Both steroids were dissolved in canola oil.

The CPP Apparatus

The CPP apparatus consisted of a gray chamber and white chamber (each 60 × 45 × 38 cm) that were separated by a clear, neutral chamber (37 × 22 × 38 cm) described previously (Meisel and Joppa, 1994). Compartments were further differentiated by corn cob bedding (Harlan 7092) in the white chamber and fine wood shavings (same as females' home cages) in the gray chamber. Sliding partitions made of the same color as the compartments were used to isolate the females during conditioning.

Pre-testing in the Preference Apparatus

Pre-tests were used to familiarize the animals to the apparatus and to determine whether the females displayed any preference for either of the two compartments. Beginning at the onset of the dark phase of the light-dark cycle, 48 h after estradiol injection, and 6 h after progesterone injection, females were placed in the clear, neutral compartment of the preference apparatus, partitions were removed, and animals were allowed to roam freely in all chambers for 10 min under supervision by an observer. The number of seconds in each compartment were recorded.

All testing was performed under fluorescent illumination within 3 h of the onset of the dark portion of the light-dark cycle. The procedure was repeated 3 times prior to conditioning, and an average of the last 2 pre-tests was used to calculate the initial preference. The apparatus was cleaned with 70% ethanol and fresh bedding was replaced after each animal was tested.

Twenty-four h food intake was measured for 4 days during pre-testing by placing approximately 20 g of food inside the home cage and weighing the food remaining 24 h later.

Conditioning in the Preference Apparatus

In conditioning sessions, subject females were paired with a stimulus in the compartment that had been less preferred in the pre-test. The mated group was placed in the previously less preferred compartment with an adult, sexually-experienced male hamster. The unmated group was never placed in a compartment with a male, but was placed alone in the previously less preferred compartment. Half the mated and half the unmated females were either fed ad libitum or food-restricted as described above. All females received the same hormonal priming prior to the conditioning test and received their conditioning sessions every 4 days.

Each conditioning session had two 10-min phases, a conditioning phase and an isolation phase. In one phase, the females were paired with the stimulus common to all members of the groups (the mated group was placed with the male stimulus, the unmated group was placed in an empty compartment). In the other phase, the females (regardless of group) were placed alone in the compartment not used in the first phase. The two phases were repeated every 4 days alternating the order of conditioning and isolation.

Post-conditioning Testing in the Preference Apparatus

Two post-conditioning tests were performed to determine whether the females had formed a CPP. One occurred after the first conditioning session and the other occurred after the fourth conditioning session.

Four days after the first conditioning session, females were hormonally-primed as described above and given a post-conditioning preference test 48 h after the estradiol injection and 6 h after the progesterone injection. Briefly, females were placed in the clear, neutral chamber and given free access to the apparatus in the absence of a male for 10 min. The amount of time spent in each compartment was recorded by an observer who was blind to prior treatments. After the initial post-test, conditioning continued every 4 days. After a total of 4 conditioning sessions, females were again given a post-test in the absence of a male and the time spent in each compartment of the apparatus recorded.

Appetitive Sexual Behavior

Four days after the last post-test, hamsters were injected with estradiol 48 h and 24 h prior to being tested for appetitive sexual behavior and aggressive behavior using a sexually-experienced male stimulus hamster. Test subjects were placed into a clear, Plexiglas cage (43 × 20 × 20 cm) containing a male and a 100 ml of a standard olfactory stimulus made from the soiled bedding collected and pooled from multiple individual, sexually-active male hamsters. Subjects were observed for 15 min with behaviors recorded every 5 sec including number of vaginal scent marks, an appetitive sex behavior known to be inhibited by food restriction (Klingerman et al., 2010).

Statistical Analysis

Time spent in the conditioned chamber was analyzed by a paired t-test to compare pre-test to post-test times. Change in body weight, food intake, and appetitive behaviors were analyzed using 2-way ANOVA with food availability and mating experience as the main effects. Post hoc tests for significant differences between groups were conducted by Duncan's Multiple Range method when the main effects were significant. Results were considered significant when $P < 0.05$.

Results

Experiment 3.1. Time Course of Effects of Food Restriction on Appetitive and Consummatory Behaviors

Sex Behaviors

Male preference on day 3 of the estrous cycle was calculated as (the amount of time females spent with a male – the amount of time spent with food) / the total time in the preference apparatus, and was inhibited by food restriction some time between day 7 and day 11 ($P < 0.05$) (Figs. 3.1A and 3.2A).

As food restriction progressed beyond 8 days, some females became anestrous, i.e., they failed to show lordosis on day 4 of the estrous cycle, even in response to a male that was freed from his wire restraint. As shown in Fig. 3.3B, 6/6, 6/6, 5/6, 4/6, 2/6, 1/6, 0/6, and 4/6 showed lordosis in response to an adult male on days 0, 4, 8, 12, 16, and 20 days after the start of food restriction, and 4 and 8 days after re-feeding. It is known from previous experiments that food-deprived or restricted female Syrian hamsters that fail to

show lordosis also show an absence of appetitive behaviors and low circulating estradiol and progesterone (Morin, 1975; Morin, 1986; Schneider et al., 2007). We wanted to know whether appetitive behaviors could be inhibited in females in which the consummatory behavior (lordosis to a free male) was not yet inhibited. It is important to use hamsters in which lordosis was not inhibited by food restriction to determine whether it is possible for food restriction to inhibit appetitive behaviors independent of lordosis and the hypothalamic-pituitary-gonadal system. Thus, in Fig. 3.1, mean male preference scores on day 3 of the estrous cycle were calculated using only females that showed lordosis to a free male on the subsequent day. Male preference on day 3 of the estrous cycle (anestrous females excluded) was significantly decreased by food restriction at 11 days after restriction and at 3 days after re-feeding ($P < 0.05$) (Fig. 3.1A). In contrast, the same females that showed decreased male preference showed no significant decrease in lordosis duration until day 20 after the start of food restriction (Fig. 3.1B).

Linear regression showed a significant portion of the variance in male preference was accounted for by variation in body weight ($r^2 = .38$, $P < 0.02$), whereas there was no significant association between lordosis duration and body weight (Fig 3.2A and 3.2B).

A similar pattern in male preference on day 3 of the cycle was apparent when all females were included in the male preference calculations (Fig. 3.3A). Food restriction decreased lordosis duration ($P < 0.05$) and this effect was magnified as restriction continued, and was reversed by 8 days of re-feeding. This was reflected in the repeated measures ANOVA as a significant main effect of food availability ($F(1,40) = 18.85$, $P < 0.002$), time ($F(4,40) = 9.27$, $P < 0.002$), and food availability x time interaction ($F(4,40)$

= 6.90, $P < 0.0003$). Furthermore, male preference was significantly lower in food-restricted females after 11 days ($P < 0.003$) and 15 days ($P < 0.02$) of food restriction and 3 days of re-feeding ($P < 0.007$) compared to ad libitum-fed hamsters (Fig. 3.3A). A similar pattern was seen when male preference was measured on day 4 of the estrous cycle, in which there was a main effect of food availability ($F(1,40) = 10.89$, $P < 0.008$), but no effect of time and no interaction (Table 3.2).

The frequency of females that showed lordosis was not significantly affected by food restriction until day 16 ($P < 0.05$), at least 5 days after male preference was affected. At 20 days after the start of restriction, all hamsters were re-fed, whether they showed lordosis or not, because we were concerned about low body weights of those hamsters that continue to show lordosis at day 20. At 4 days after re-feeding, none of the food-restricted females showed lordosis. This has been observed previously when very lean animals were restricted on day 4 of the cycle, but did not recover estrous cyclicity by 4 days of re-feeding (Schneider, unpublished data). By 8 days of re-feeding, the majority of females showed lordosis, and the frequency of lordosis did not differ between food-restricted and ad libitum-fed females (Fig. 3.3B).

The majority of vaginal scent marks were produced on day 3 of the estrous cycle, the day before behavioral estrous (Table 3.1). Ad libitum-fed females tended to show higher levels of vaginal marking compared to restricted, but this difference was only significant on day 11 of restriction ($P < 0.03$). Repeated measures ANOVA showed no main effect of food availability or time, but there was a significant interaction between time and food availability ($F(4,40) = 49.61$, $P < 0.03$) on the number of vaginal marks

per 15 min. In the present experiment, the rate of vaginal scent marking did not significantly increase after re-feeding.

Food-restricted females showed significantly fewer flank marks compared to ad libitum-fed females after 11 days of food restriction ($P < 0.04$) (Table 3.1).

Ingestive Behavior

Food hoarding (day 3 of the estrous cycle) increased in food-restricted females as reflected by the repeated measures ANOVA, which showed a significant main effect of food availability ($F(1,40) = 7.35$, $P < 0.02$), time ($F(4, 40) = 4.71$, $P < 0.003$), and an interaction between food availability and time ($F(4,40) = 6.40$, $P < 0.0004$). The amount of food hoarded on day 3 was significantly greater in food-restricted hamsters compared to hamsters fed ad libitum after 11 days ($P < 0.04$) and 15 days ($P < 0.01$) of food restriction and remained increased through 3 days of re-feeding ($P < 0.03$) (Fig. 3.4A). Similarly, for food hoarding on day 4 of the estrous cycle, there was a significant main effect of time ($F(4,40) = 3.24$, $P < 0.02$) and an interaction between food availability and time ($F(4,40) = 3.61$, $P < 0.01$) (Table 3.2).

There was no effect of food restriction on food intake on day 3 of the cycle (Fig. 3.4B). On day 3 of the cycle, there was a main effect of time on the amount of food eaten ($F(4,40) = 2.74$, $P < 0.04$), but there was no interaction between food availability and time (Table 3.1). Food-restricted females never differed significantly in their food intake from ad libitum-fed females on any day of day 3 testing.

On day 4 of the estrous cycle, most ad libitum-fed females went directly to the male and spent all of their time with males, whereas late in food restriction, some females made brief visits to the food box, particularly those who were anestrous. Food intake did not differ significantly between food-restricted and ad libitum-fed females on any day (Table 3.2). However, on day 4 of the estrous cycle, there were main effects of food availability ($F(1,28) = 27.90$, $P < 0.001$) and time ($F(4,28) = 3.83$, $P < 0.01$) and an interaction between food availability and time ($F(4,28) = 2.76$, $P < 0.05$) on food intake (Table 3.2).

Body Weight

Female hamsters food-restricted for 16 days lost weight (an average of -31.5 ± 5.58 g S.E.M.) whereas females fed ad libitum gained weight (+10.5 ± 3.28 g S.E.M.) and the change in body weight was significant ($F(1,12) = 47.20$, $P < 0.0001$) (Fig. 5). After eight days of re-feeding, the previously food-restricted hamsters increased body weight, but remained significantly lower than ad libitum-fed hamsters ($P < 0.018$) (Fig. 3.5).

Experiment 3.2. Mating-induced Neural Activation in Food-restricted and Ad libitum-fed Females

Lordosis Frequency and Duration

Ten days of food restriction at 75% of ad libitum intake resulted in significant body weight loss ($F(1,22) = 37.08$, $P < 0.0001$) (Fig. 3.5), and did not result in significant

differences in the frequency of or duration of lordosis (Fig. 3.6) compared to ad libitum-fed hamsters. Male hit rate was determined by dividing the number of intromissions/ejaculations by the number of mounts. Males that mated with food-restricted females did not show a significantly different hit rate or number of sexual attempts than those that mated with ad libitum-fed female hamsters (Fig. 3.6).

c-Fos Immunoreactivity

For c-Fos-IR in the NAc core, there was a significant main effect of mating ($F(1,19) = 9.45$, $P < 0.006$), but no significant effect of food treatment and no interaction between food treatment and mating (Fig. 3.7). Analysis of the NAc shell yielded similar results with a significant main effect of mating ($F(1,19) = 10.42$, $P < 0.004$) and no significant effect of food treatment and no significant interaction between food treatment and mating (Fig. 3.7).

For c-Fos-IR in the lateral VMH there was a significant main effect of mating (mating experience increased c-Fos-IR) ($F(1,19) = 22.0$, $P < 0.0002$) but no significant effect of food treatment and no significant interaction between food treatment and mating (Fig. 3.8).

Within the medial VMH, there was no significant main effect of mating, food treatment or interaction between food treatment and mating. c-Fos-IR in the VMH did not differ between food-restricted and ad libitum-fed hamsters after mating at 10 days of restriction in either the lateral or medial VMH (Fig. 3.8).

For c-Fos-IR in the Arc, the general pattern was different than the VMH, with food-restricted females always showing somewhat higher levels than ad libitum-fed, and mated lower than non-mated females, but there was no significant main effect of mating or food treatment and no significant interaction between food treatment and mating (Fig. 3.8).

For c-Fos-IR in the MeA, there was a significant main effect of mating ($F(1,19) = 11.07$, $P < 0.004$) and no significant effect of food treatment and no significant food treatment by mating interaction, although it appeared that the mating response in food-restricted females might be exaggerated compared to that of ad libitum-fed females (Fig. 3.9).

For c-Fos-IR in the PVN there was no significant main effect of food treatment, mating, and no significant interaction between food treatment and mating (Fig. 3.10).

Experiment 3.3. Effects of Energy Restriction on Formation of a Conditioned Place Preference to Mating

Conditioned Place Preference

Both food-restricted and ad libitum-fed females developed a CPP to mating after 4 conditioning sessions (Fig. 3.11). Food-restricted and ad libitum-fed hamsters did not form a preference to mating after 1 conditioning session ($P < 0.26$ and $P < 0.15$ respectively). However, after 4 conditioning sessions, both food-restricted and ad libitum-fed hamsters formed a preference to mating ($P < 0.004$ and $P < 0.03$). There were also significant differences between 1 and 4 mating sessions for hamsters food-

restricted and fed ad libitum ($P < 0.03$ and $P < 0.05$ respectively). Food-restricted and ad libitum-fed females conditioned to an empty box failed to form a preference after 1 ($P < 0.61$ and $P < 0.56$ respectively) or 4 conditioning sessions ($P < 0.84$ and $P < 0.26$ respectively).

Food Intake

Cumulative food intake (Fig. 3.12) differed significantly between food-restricted and ad libitum-fed hamsters by the first conditioning session ($F(1,20) = 15.43$, $P < 0.0008$) and this difference continued through the last post-test ($F(1,20) = 10.85$, $P < 0.0036$) and appetitive sex test (data not shown).

Body Weight

Both food-restricted and ad libitum-fed hamsters lost weight throughout Experiment 3.3 (Fig. 3.5), however, hamsters that were food-restricted lost significantly more weight than hamsters fed ad libitum. By the first conditioning session (8 days of restriction) food-restricted hamsters lost significantly more weight (weights subtracted from pre-test) than hamsters fed ad libitum ($F(1,20) = 13.31$, $P < 0.002$) and this difference remained throughout the last post-test (30 days of food restriction) and appetitive sex test ($F(1,20) = 8.79$, $P < 0.008$).

Appetitive Sex Test

Similar to Experiment 3.1, hamsters that were food-restricted were less sexually-motivated than hamsters fed ad libitum. For example, food-restricted hamsters produced significantly less vaginal marks (Fig. 3.13) than hamsters fed ad libitum ($F(1,20) = 4.42$, $P < 0.05$) and there was no effect of prior mating experience obtained during conditioning ($F(1,20) = 0.67$, $P < 0.42$) or an interaction between food and mating ($F(1,20) = 1.10$, $P < 0.31$). Although not significant, there was a tendency for food-restricted hamsters to have a longer latency to vaginal mark ($F(1,20) = 3.61$, $P < 0.07$) and shorter latency to aggression ($F(1,20) = 3.31$, $P < 0.08$), compared to hamsters fed ad libitum (data not shown). Also, twice as many females fed ad libitum (8 hamsters) allowed mounting attempts by a male compared to food-restricted hamsters (4 hamsters).

Discussion

The main findings from these experiments were threefold. First, 25% food restriction for greater than 7 but less than 12 days significantly decreased the motivation for sex without a significant decrease in sexual performance in the majority of females (Figs. 3.1 and 3.2). These results are consistent with an earlier study in which 8 days of 25% food restriction significantly decreased sexual motivation but not performance (Klingerman et al., 2010). Second, ten days of 25% food restriction failed to prevent or significantly attenuate lordosis duration, copulatory interactions with males, or mating-induced neural activation in the NAc, VMH, PVN, MeA, or Arc in Experiment 3.2 (Figs. 3.7-10). Third, between 8 and 30 days of 25% food restriction significantly decreased vaginal marking, but failed to effect formation of a CPP to mating in Experiment 3.3.

The results of Experiment 3.1 are consistent with previous studies demonstrating that the mechanisms that control the motivation to engage in sex and/or ingestive behavior are more sensitive to the effects of energy availability than are the mechanisms that control copulatory performance (Klingerman et al., 2010; Schneider et al., 2007). Specifically, after 11 days of food restriction, appetitive sex behaviors were significantly lower and appetitive ingestive behaviors were significantly higher in food-restricted compared to ad libitum fed females (Figs. 3.1A and 3.4A), even in females that showed lordosis on the next day of the same estrous cycle (Fig. 3.1B). The 12-day food-restricted females that showed lordosis did not differ significantly from ad libitum-fed females in lordosis duration (Fig. 3.1B). Also, on day 12 of food restriction, only two of six food-restricted females failed to show lordosis when the male was free (Fig. 3.3B). Thus, although copulatory performance can be affected by 12 days of restriction in some females, inhibition of lordosis and the underlying HPG system is not required in order for this level of food restriction to inhibit appetitive sex behaviors and increase appetitive ingestive behaviors on day 11. In addition, there was a significant positive correlation between male preference on day 3 of the estrous cycle and body weight, but the correlation between lordosis duration on day 4 of the estrous cycle and body weight was not significant (Fig. 3.5). Together, these results reveal and crystallize an important interaction between energy availability, male stimuli and ovarian steroids in setting behavioral priorities that is not apparent by limiting observation to consummatory behaviors such as lordosis and food intake.

The effects of this mild food restriction are not explained by decreases in circulating ovarian steroid concentrations because in Experiment 3.1 appetitive behavior was inhibited even in females that showed lordosis, a behavior dependent upon high circulating levels of estradiol and progesterone. In addition, in Experiment 3.3 vaginal scent marking was significantly decreased by food restriction despite treatment with estradiol (Fig. 3.13). In other experiments, two days of complete food deprivation significantly decreased vaginal scent marking by estrous-cycling Syrian hamsters, even though these hamsters did not show food deprivation-induced anestrus or a significant decrease in circulating estradiol concentrations (Schneider et al., 2007). In other experiments, neither estradiol nor progesterone concentrations were significantly lower after 4, 8 or 12 days of 75% food restriction (Klingerman et al., 2011). Finally, significant effects on appetitive sex and ingestive behavior were seen when ovariectomized females were food restricted to 75% of their ad libitum intake for 8 days treated and with estrous-inducing doses of exogenous estradiol and progesterone (Klingerman et al., 2010).

It is likely that the effects of food restriction on appetitive behaviors involves decreased sensitivity or responsiveness to estradiol because effects of energy availability occur prior to endogenous changes in circulating ovarian steroids and in spite of exogenous treatment with ovarian steroids. Energetic effects on neural estrogen receptors have been noted in the literature, but the energetic challenges were far more severe and inhibited both lordosis and the hypothalamic-pituitary-gonadal system. In earlier studies, food deprivation in lean female hamsters (less than 100 g in body weight) inhibited

follicle development and ovarian steroid secretion, induced anestrous, and decreased the number of cells immunoreactive for estrogen receptor (ER-IR) in the VMH in hamsters (Li et al., 1994; Morin, 1986), and induced hypogonadotropism in other species (Bronson, 1988; I'Anson et al., 2000; I'Anson et al., 2003). Although ER-IR is lower in the VMH and higher in the PVN and caudal preoptic area (POA) after 48 or more hours of total food deprivation in lean hamsters (less than 120 g) (Li et al., 1994), the effects of the current food-restriction paradigm (25% food restriction in 140 g hamsters) on ER-IR remain to be investigated.

An additional new finding from the present experiment was that a similar level and duration of food restriction relative to that used in Experiment 3.1 did not have a significant effect on either the females' lordosis duration, or on the naïve stimulus males' hit rate (number of intromissions per number of mounts) in Experiment 3.2 (Fig. 3.6). Other experiments show that when sexually-naïve male hamsters mate with sexually-experienced females, they achieve a significantly greater hit rate than when they mate with sexually-naïve females. Furthermore, male hit rate is improved when their female mating partners receive treatments that increase the expression of ΔFosB in the NAc of females (Hedges et al., 2009). Given these results, it might be expected that the females' energetic condition might also influence their ability to improve male hit rate. This idea was refuted because there was no significant difference in hit rate between those males that mated with females that were food-restricted and those that mated with females that were fed ad libitum (Fig. 3.6).

Having identified a duration during which 25% food restriction blocks appetitive but not consummatory behaviors in Experiment 3.1, Experiment 3.2 was designed to test the idea that food-restriction-induced changes in appetitive behaviors might be dissociated from changes that result from the rewarding consequences of copulation. The NAc was of particular interest because a 10-min period of sexual experience increases neural activation and dopamine release in the NAc, and the effect is exaggerated in females with prior sexual experience (Bradley and Meisel, 2001; Kohlert and Meisel, 1999). Furthermore, just as the effects of 25% food restriction decreased appetitive behaviors prior to consummatory behaviors, treatment with a dopamine receptor antagonist blocked the formation of a conditioned place preference after sexual experience without significant effects on lordosis (Meisel et al., 1996). Would food restriction also inhibit copulation-induced increases in NAc neural activation in the same way that it inhibited appetitive behavior, or would appetitive behavior be dissociated from the rewarding aspects of copulation? A third possible outcome would be that food restriction would actually enhance NAc neural activation, similar to the food restriction-induced sensitization to the effects of drugs of abuse (Carr, 2002; Pan et al., 2006). In Experiments 3.2 and 3.3, inhibition of appetitive behavior was dissociated from mechanisms that mediate the rewarding consequences of copulation because both ad libitum-fed and food-restricted females showed mating-induced increases in neural activation in the NAc, similar to the pattern of neural activation found in the VMH and MeA (Figs. 3.7-9). Furthermore, food-restricted and ad libitum-fed females both showed evidence that they experienced a reward during copulation, because these groups did not

differ significantly in the formation of a CPP (Fig. 3.11), despite the fact that food-restricted females decreased appetitive sex behavior (Fig. 3.13).

Whereas mating experience increased neural activation in the NAc, VMH and MeA (Figs. 3.8 and 3.9), a different pattern of neural activation was seen in the Arc and PVN (Fig.3.8 and 3.10). In the Arc, mating experience did not stimulate c-Fos expression. In the Arc, neural activation in the food-restricted group was always slightly higher than in females fed ad libitum, and these effects were not significant (Fig. 3.8). Similarly, in the PVN, effects of mating and food regimen were not significant (Fig. 3.10). Together these results suggest that the effects of energy availability on sexual motivation might be mediated by mechanisms that are not reflected by mating-induced neural activation in the NAc. Changes in these latter areas would be consistent with their high concentration of corticotropin releasing hormone, urocortin, leptin receptors, neuropeptide Y/agouti-related protein, and proopiomelanocortin neurons and their putative roles in response to energy deficits (Backholer et al., 2009; Baldo et al., 2003; Bentley et al., 2006; Hakansson et al., 1998; Jones et al., 2004; Mercer et al., 1998; Qi et al., 2009; Seymour et al., 2005; Tartaglia et al., 1995) but 25% food restriction might not be enough to significantly increase activation of these cells.

The present experiment addressed only the idea that energy deficits decrease the rewarding aspects of a mating experience that involves mounting, intromission and ejaculation. A separate question is whether precopulatory, appetitive behaviors are reinforcing in ad libitum-fed females (whether they can serve as a reinforcing stimulus in a CPP paradigm or whether these experiences can sensitize the dopamine transmission or

neural activation in the NAc) and if so, whether the reinforcing aspects of behavior are susceptible to energy deficits. The answer to these questions with regard to ad libitum-fed females are unknown and thus, there is currently no basis for comparisons to food-restricted females.

In summary, the present experiments confirm that 25% food restriction can be used to reliably decrease appetitive aspects of sex and ingestive behavior without affecting consummatory behavior in the majority of female hamsters and mating reward. The behavioral results suggest that hamster precopulatory behaviors, including the choice between courtship and hoarding, represent an important locus of effect of energy availability on reproduction. This is a useful paradigm for elucidating the functional significance of neuropeptides and neurohormones previously thought to be important for body weight regulation, but which might have evolved to orchestrate the appetites for food and sex. Furthermore, the choice between engaging in sex or ingestive behaviors might be the point at which the reproductive outcome (and Darwinian fitness) is decided, and thus, the mechanisms that control this choice might be the mechanisms that have been under selection. If so, it will be important to reexamine the assertions that have come from many years of studying food intake in animals in an enclosed space with no opposite-sex conspecifics available. For example, if the adaptive function of so-called ‘satiety peptides,’ such as leptin and estradiol, has been to temporarily decrease ingestive behaviors and to stimulate reproductive behaviors, it might be imprudent to expect that these hormones limit anticipatory shopping, hoarding and body weight gain.

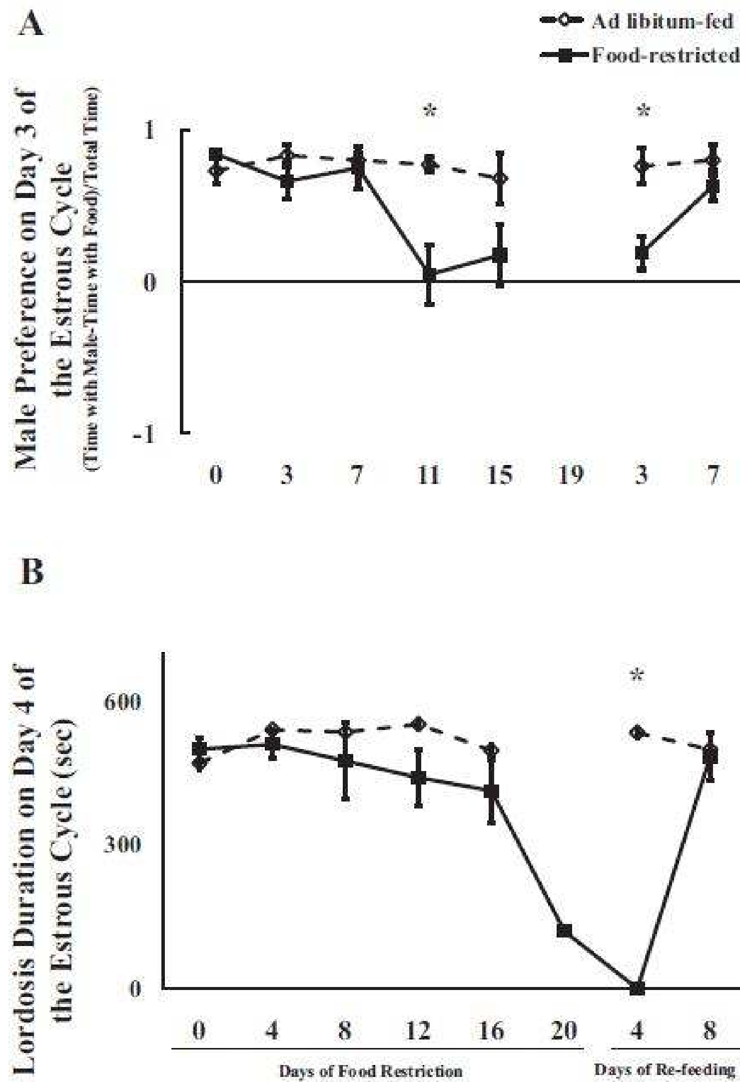
Table 3.1
Day 3 of the estrous cycle.

	Length of Restriction (d)					Length of Re-feeding (d)	
	0	3	7	11	15	3	7
Number of Vaginal Marks							
Ad libitum	2.4 ± 1.1	3.8 ± 1.7	4.1 ± 1.3	9.3 ± 2.9	3.5 ± 1.4	2.0 ± 2.0	9.8 ± 2.4
Food-restricted	3.2 ± 1.7	5.0 ± 1.6	1.7 ± 0.8	1.0 ± 0.8*	1.8 ± 1.1	1.7 ± 1.3	5.0 ± 2.2
Number of Flank Marks							
Ad libitum	3.6 ± 1.5	7.1 ± 2.4	8.8 ± 2.6	7.0 ± 2.6	3.9 ± 1.4	6.5 ± 4.0	12.5 ± 2.1
Food-restricted	5.0 ± 1.8	3.0 ± 1.7	4.3 ± 3.2	0.3 ± 0.3*	1.0 ± 0.6	3.5 ± 2.6	12.8 ± 3.8

Table 3.2
Day 4 of the estrous cycle.

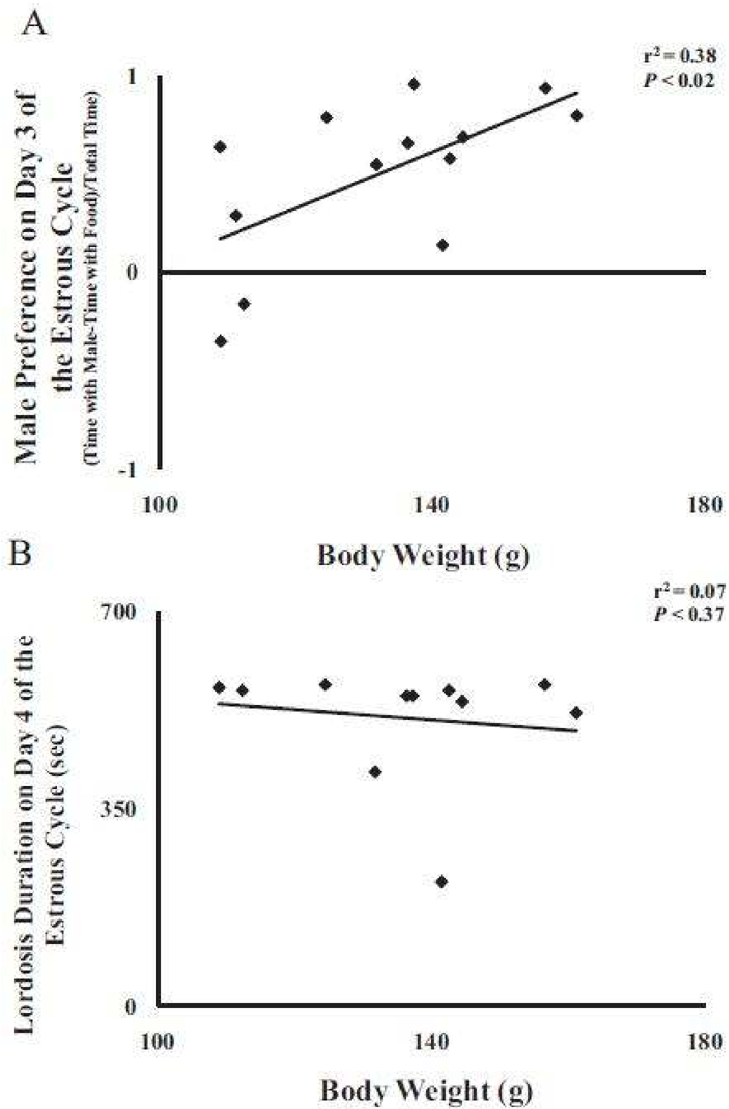
	Length of Restriction (d)					Length of Re-feeding (d)	
	0	4	8	12	16	4	8
90-min Food Hoarding (g)							
Ad libitum	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Food-restricted	2.7 ± 2.7	0 ± 0	40.1 ± 25.2	50.6 ± 31.0	42.7 ± 28.6	65.3 ± 29.0	36.9 ± 23.5
90-min Food Intake (g)							
Ad libitum	0.6 ± 0.4	0.2 ± 0.1	0.5 ± 0.2	0.8 ± 0.5	1.0 ± 0.3	2.8 ± 2.2	0.4 ± 0.1
Food-restricted	0.8 ± 0.5	2.4 ± 0.4	0.9 ± 0.3	2.2 ± 0.9	2.5 ± 1.1	0.9 ± 0.5	0.8 ± 0.3
Male Preference for All Females (Time with Male - Time with Food) / Total Time							
Ad libitum	0.92 ± 0.01	0.95 ± 0.01	0.97 ± 0	0.96 ± 0.01	0.94 ± 0.02	0.95 ± 0.01	0.96 ± 0.01
Food-restricted	0.94 ± 0.01	0.94 ± 0.03	0.72 ± 0.16	0.51 ± 0.21*	0.61 ± 0.22	0.67 ± 0.10*	0.82 ± 0.09
Male Preference Without Anestrous Females (Time with Male - Time with Food) / Total Time							
Ad libitum	0.92 ± 0.01	0.95 ± 0.01	0.97 ± 0	0.96 ± 0.01	0.94 ± 0.02	0.95 ± 0.01	0.96 ± 0.01
Food-restricted	0.94 ± 0.01	0.94 ± 0.03	0.80 ± 0.17	0.69 ± 0.27	0.95 ± 0.01	0 ± 0*	0.86 ± 0.11

Figure 3.1



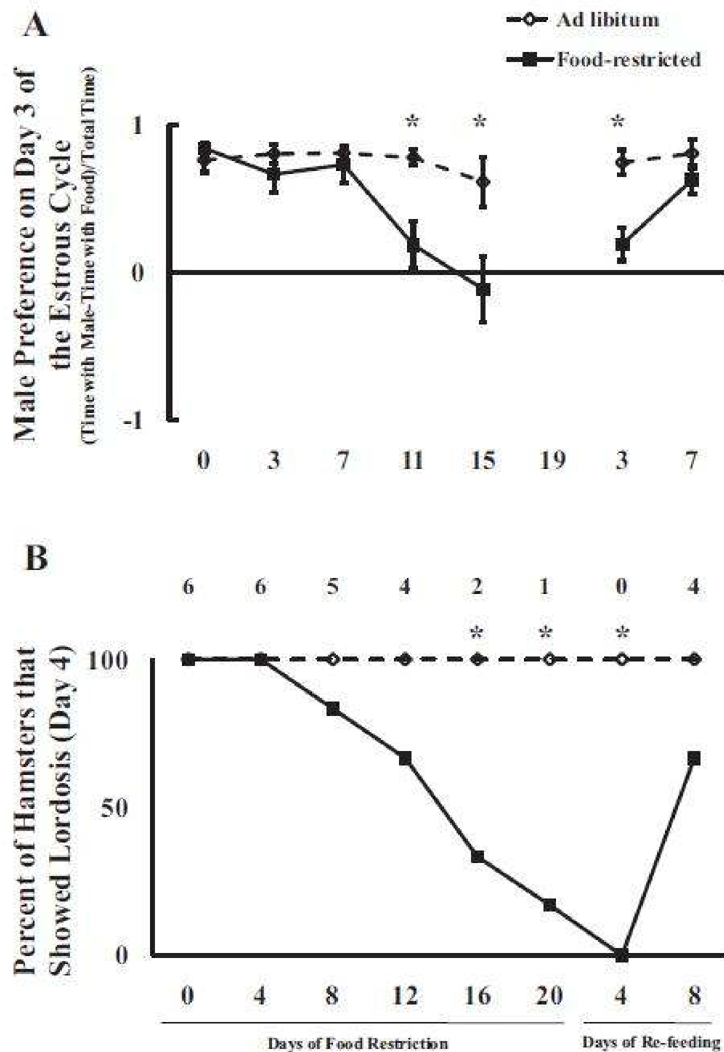
Mean and standard error of the mean for A) male preference (time with male – time with food) /total time on day 3 of the estrous cycle, and B) lordosis duration on day 4 of the estrous cycle. Only females that showed lordosis on day 4 of the same estrous cycle are included. Females were fed ad libitum or food-restricted to 75% of their ad libitum intake until they stopped showing lordosis, at which point they were re-fed ad libitum. * Significant difference between food-restricted and ad libitum-fed hamsters at $P < 0.05$.

Figure 3.2



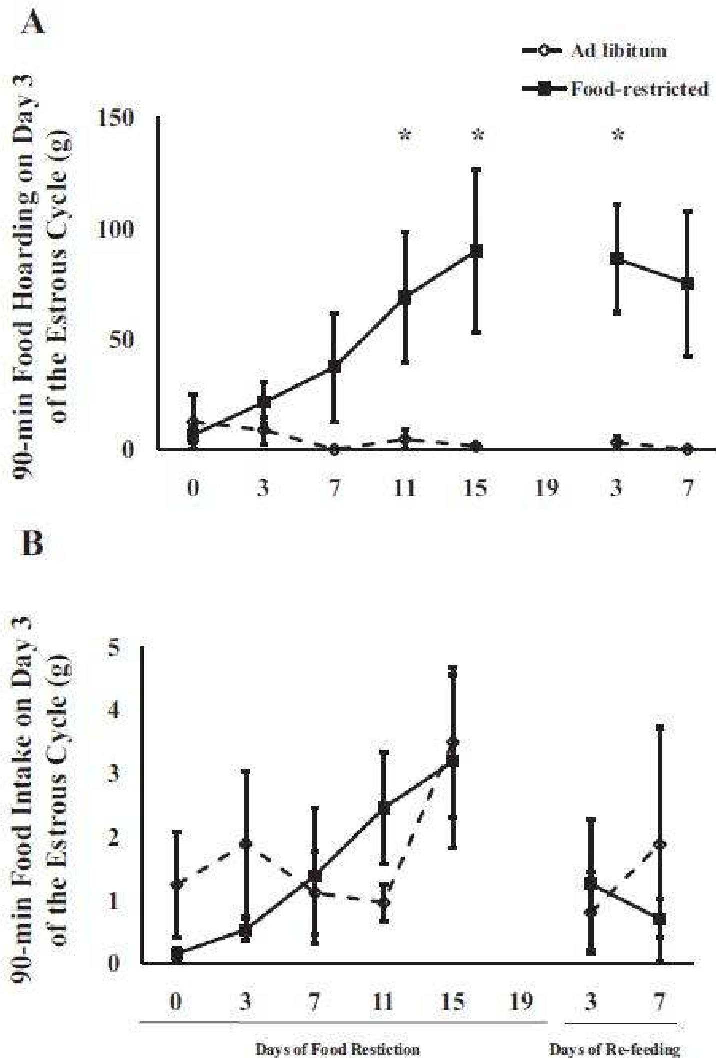
Regression of A) male preference, and B) lordosis on body weight at the time of behavioral testing. Male preference was calculated as (time with a male – time with food) /total time on day 3 of the estrous cycle.

Figure 3.3



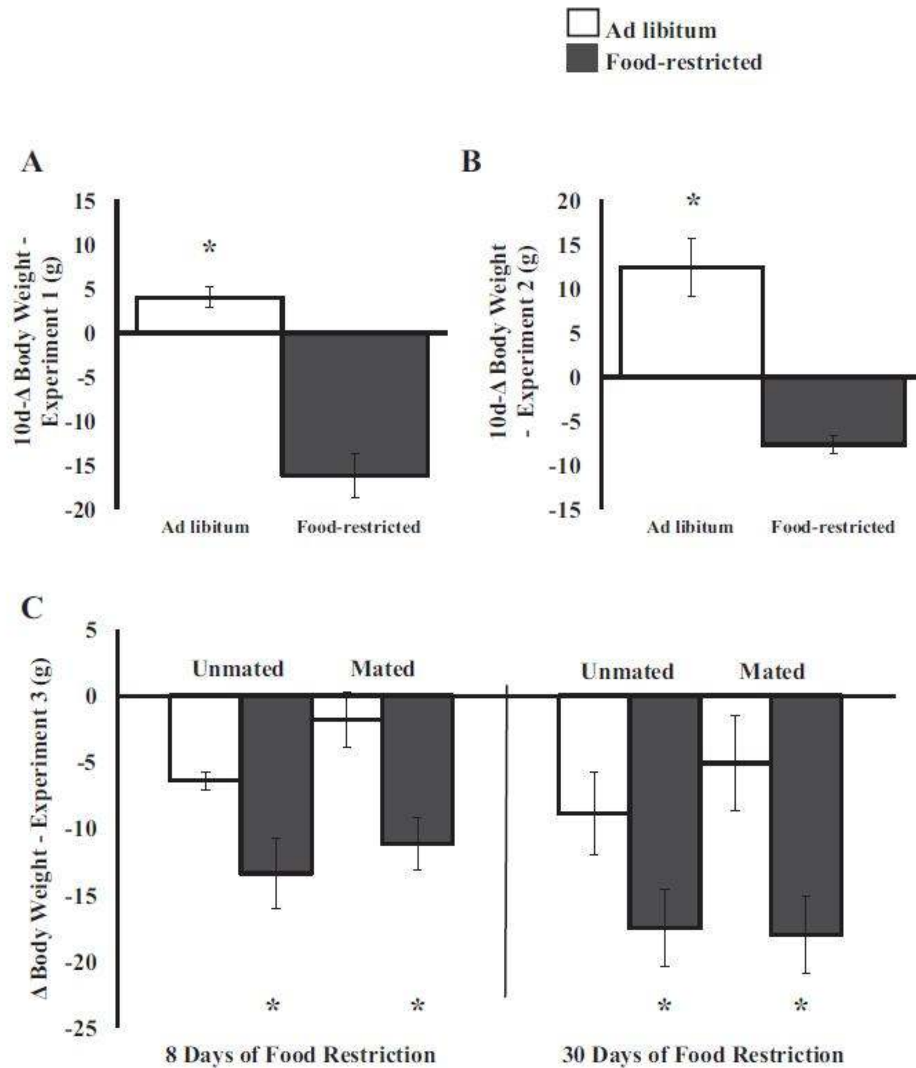
Mean and standard error of the mean for A) male preference (time with male – time with food)/total time for all females on day 3 of the estrous cycle including those that did not show lordosis (those that did not show lordosis received a score of 0), and B) the frequency of females that showed lordosis. Females were fed ad libitum or food-restricted to 75% of their ad libitum intake until they stopped showing lordosis, at which point they were re-fed ad libitum. * Significant difference between food-restricted and ad libitum-fed hamsters at $P < 0.05$.

Figure 3.4



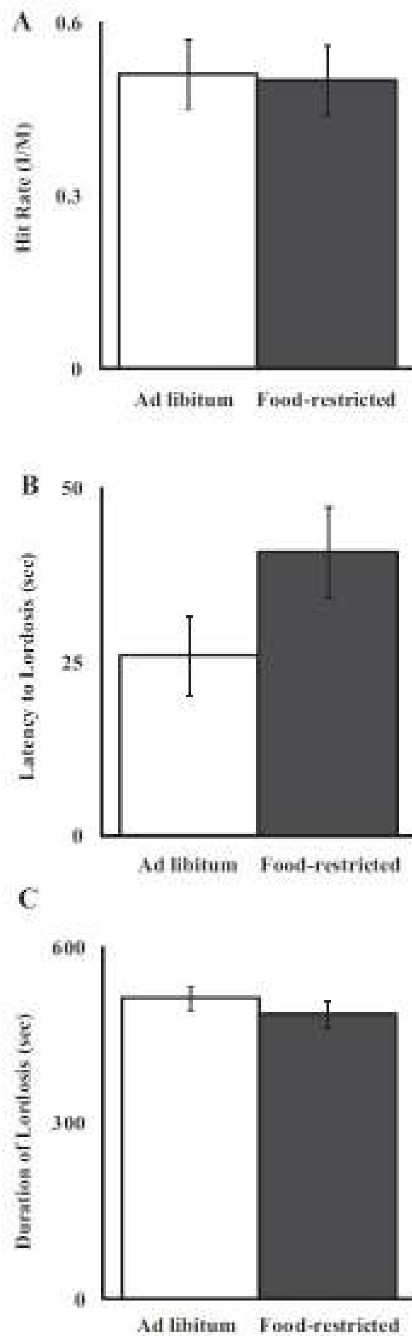
Mean and standard error of the mean for A) 90-min food hoarding, and B) 90-min food intake on day 3 of the estrous cycle. Females were fed ad libitum or food-restricted to 75% of their ad libitum intake until they stopped showing lordosis, at which point they were re-fed ad libitum. Hamsters were tested for food hoarding and intake at the same time they had access to an adult, sexually experienced male restrained behind a wire barrier. * Significant difference between food-restricted and ad libitum-fed hamsters by $P < 0.05$.

Figure 3.5



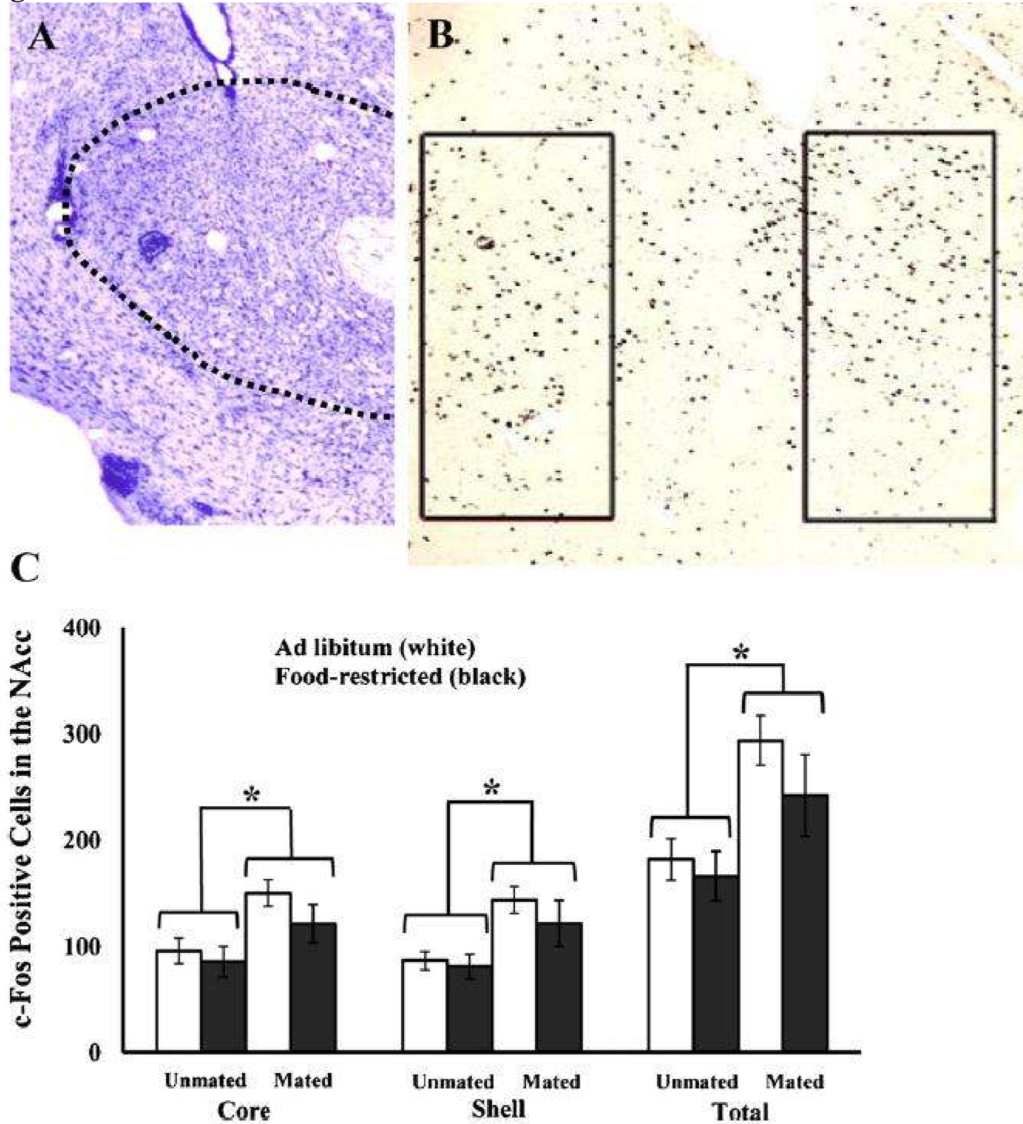
Mean and standard error of the mean for the change in body weight from the start of restriction to day 10 in females from A) Experiment 3.1 and B) Experiment 3.2. C) Mean and standard error of the mean for the change in body weight from the start of restriction to the first conditioning session (8 days of restriction) and from the start of the experiment to the last post-conditioning test (30 days of restriction) from Experiment 3.3. Females received a conditioning session or a post-test every 4 days. * Significantly different at $P < 0.05$.

Figure 3.6



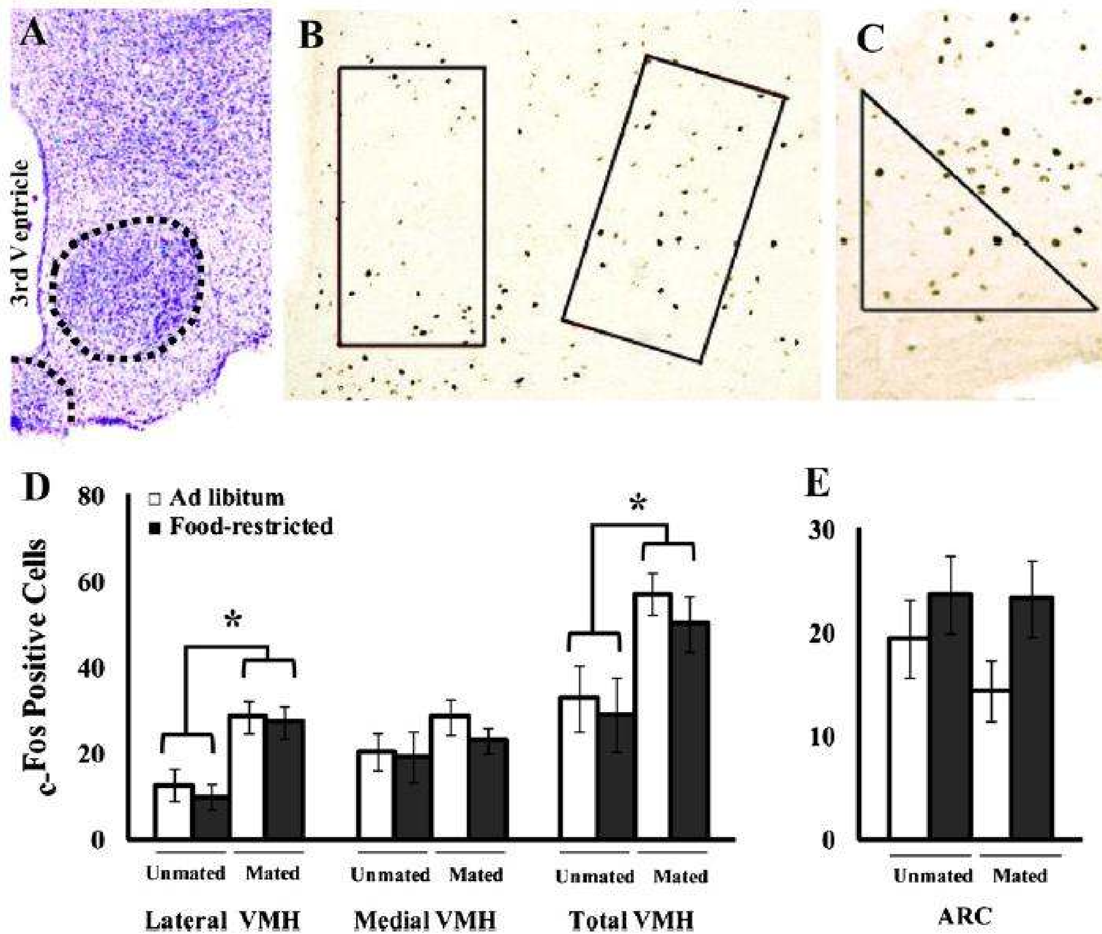
Mean and standard error of the mean for A) hit rate (calculated as the number of intromissions/the number of mounts), B) latency to lordosis, and C) lordosis duration. * Significantly different at $P < 0.05$.

Figure 3.7



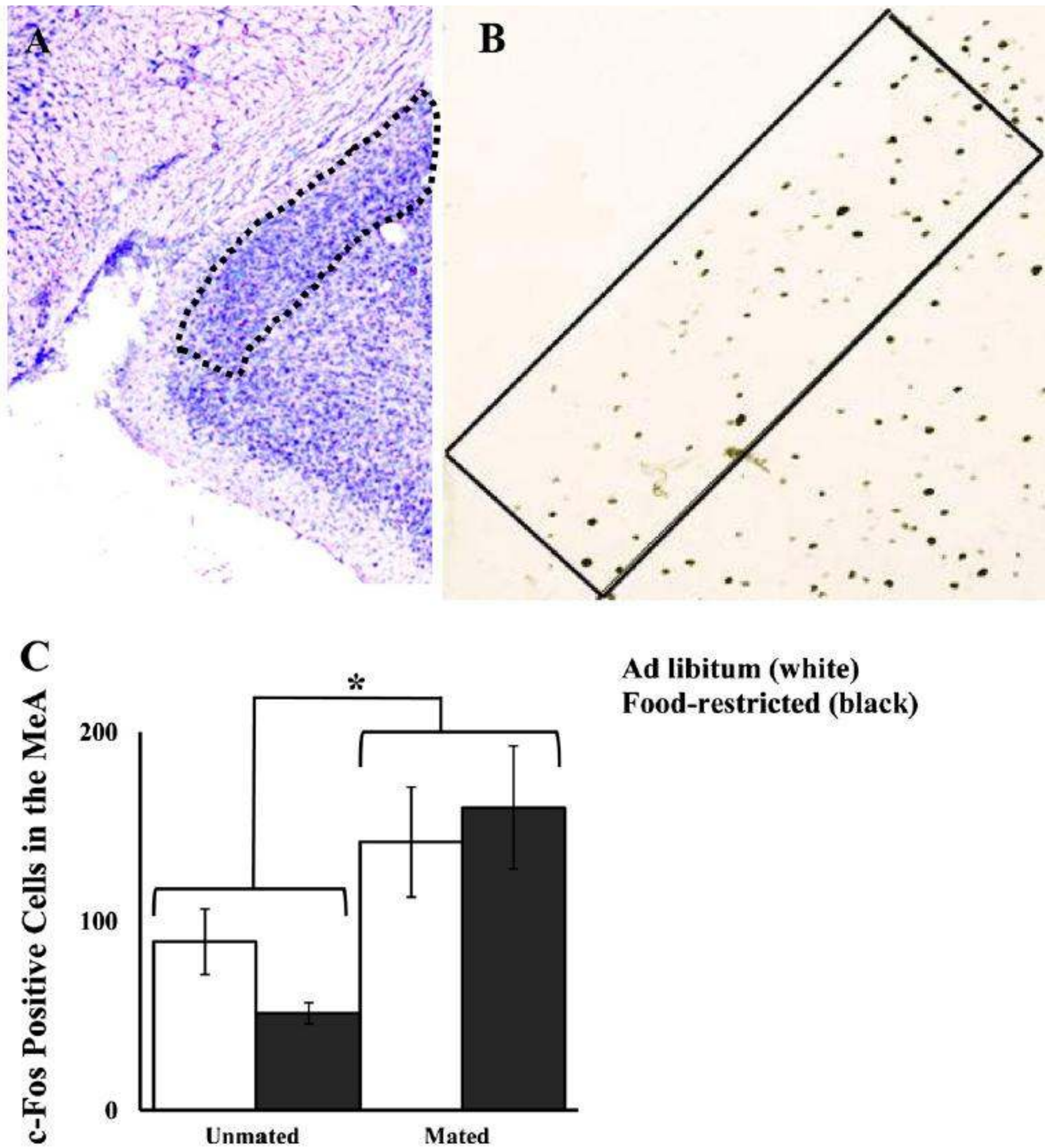
Representative cresyl violet A) and DAB stained B) images of the NAc. Food-restricted hamsters were fed 75% of their ad libitum intake for 10 days. Mated hamsters were given a 10 min mating test with a sexually naïve male hamster and unmated hamsters had no contact with a male prior to sacrifice 1 h later. C) Mean and standard error of the mean for c-Fos immunoreactivity in the NAc. * Mated hamsters significantly different from unmated hamsters at $P < 0.05$.

Figure 3.8



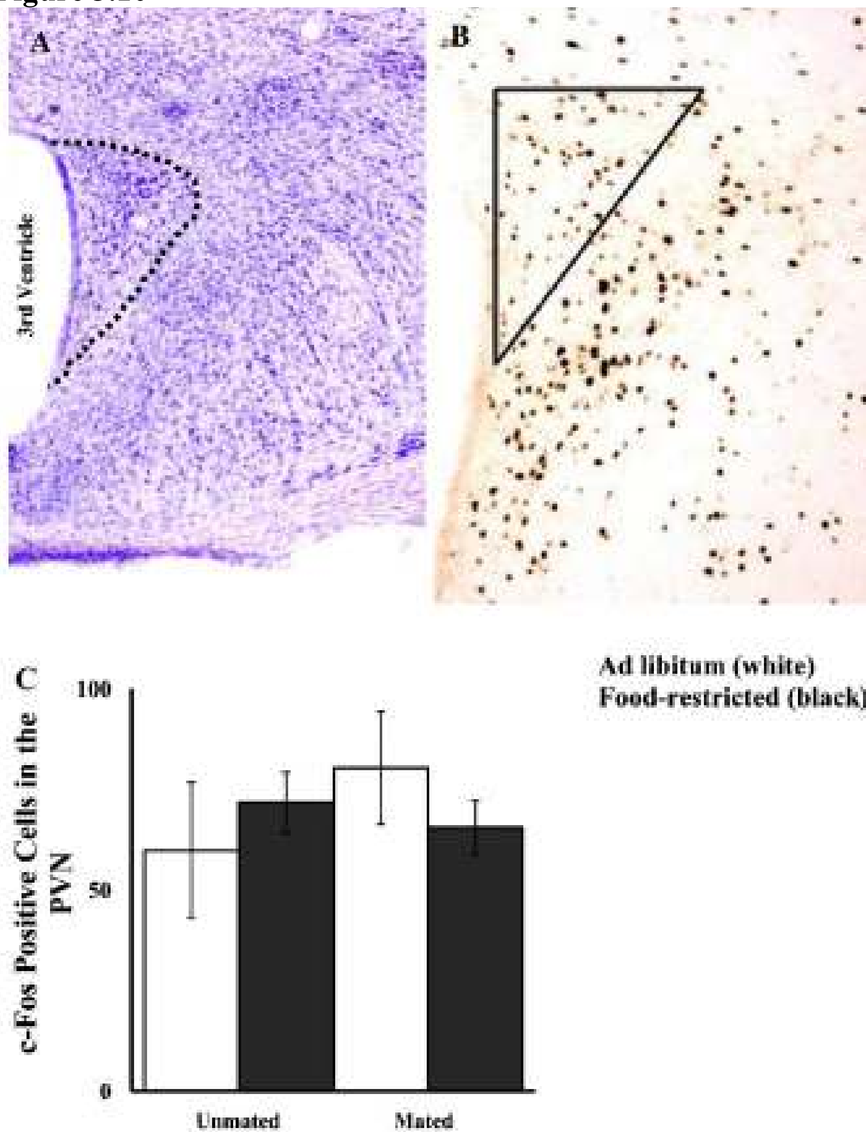
Representative cresyl violet A) and DAB stained (B and C) images of the VMH and Arc. Food-restricted hamsters were fed 75% of their ad libitum intake for 10 days. Mated hamsters were given a 10-min mating test with a sexually naïve male hamster and unmated hamsters had no contact with a male prior to sacrifice 1 h later. Mean and standard error of the mean for c-Fos immunoreactivity in the D) lateral VMH and E) arcuate nucleus. * Mated hamsters significantly different from unmated hamsters at $P < 0.05$.

Figure 3.9



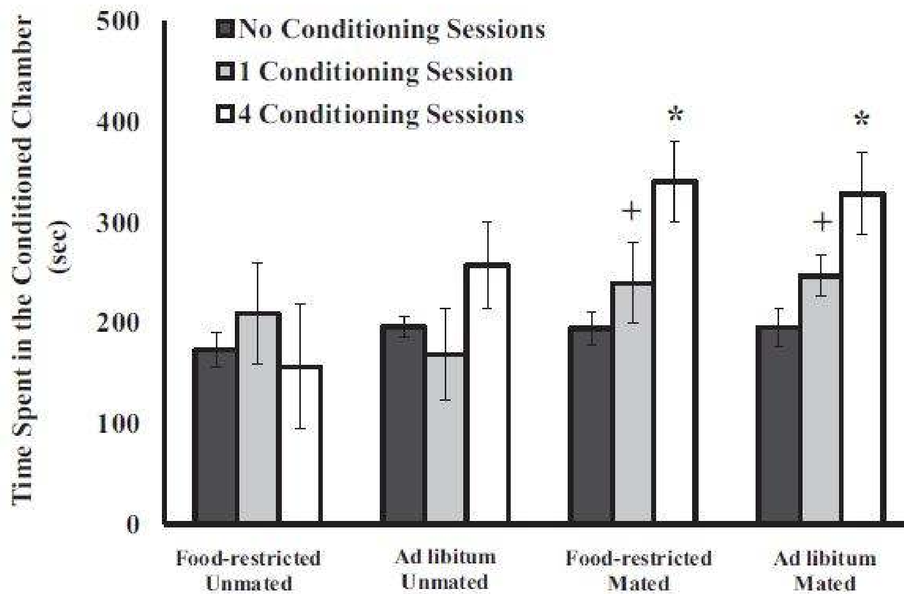
Representative cresyl violet A) and DAB stained B) images of the MeA. Food-restricted hamsters were fed 75% of their ad libitum intake for 10 days. Mated hamsters were given a 10-min mating test with a sexually-naïve male hamster and unmated hamsters had no contact with a male prior to sacrifice 1 h later. C) Mean and standard error of the mean for c-Fos immunoreactivity in the MeA.

Figure 3.10



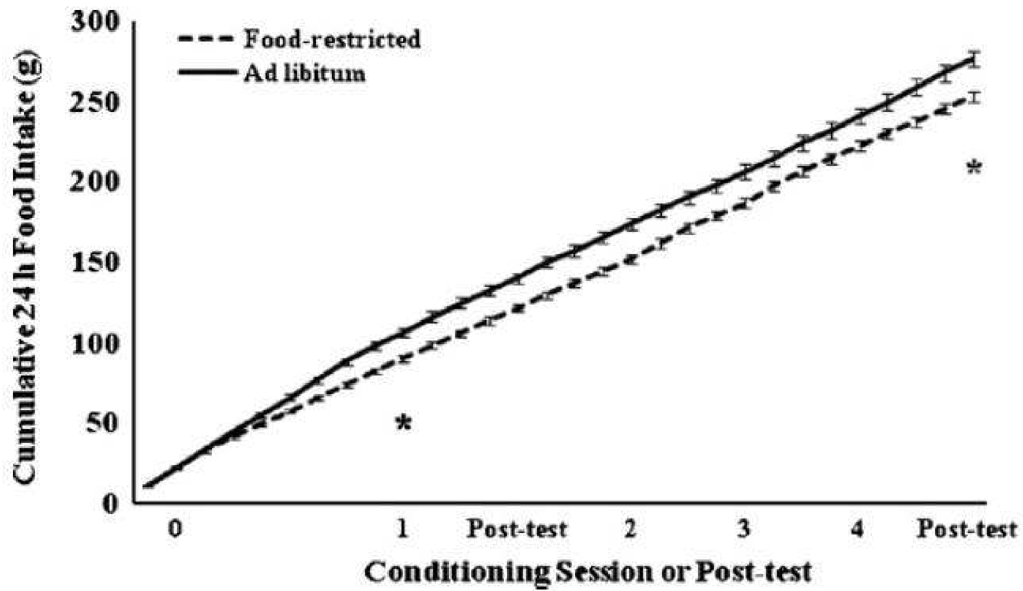
Representative cresyl violet A) and DAB stained B) images of the PVN. Food-restricted hamsters were fed 75% of their ad libitum intake for 10 days. Mated hamsters were given a 10-min mating test with a sexually naïve male hamster and unmated hamsters had no contact with a male prior to sacrifice 1 h later. C) Mean and standard error of the mean for c-Fos immunoreactivity in the PVN. * Mated hamsters significantly different from unmated hamsters at $P < 0.05$.

Figure 3.11



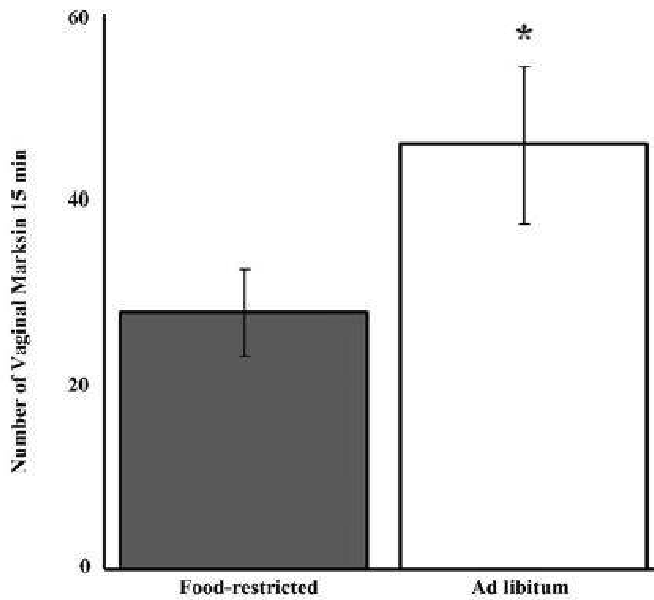
Mean and standard error of the mean of time spent in the conditioned chamber of the CPP apparatus in hamsters tested after 1, 2, or 4 post-conditioning tests. Females were all ovariectomized and treated with estradiol and progesterone and half were either fed ad libitum or food-restricted to about 75% of their ad libitum intake beginning 8 days prior to conditioning through the last post-test. Half of each food group was either conditioned with a male (mated) or conditioned with the empty compartment (unmated). During post-conditioning tests, female subjects had a choice between a chamber where 10 min of copulatory experiment had occurred (conditioned compartment) or where no male had been present (unconditioned compartment). Females were tested for their preference for either the conditioned or unconditioned compartment after 0, 1, or 4 conditioning tests. + 1 conditioning session significantly different from 4 conditioning tests by $P < 0.05$. * 0 conditioning sessions (pre-test) significantly different from 4 conditioning sessions at $P < 0.05$.

Figure 3.12



Mean and standard error of the mean of cumulative food intake over the course of Experiment 3.3. Females were food-restricted to 75% of their ad libitum food intake or fed ad libitum for 8 days prior to the first CPP conditioning trial and throughout conditioning. * Cumulative food intake of food-restricted hamsters was significantly less than that of ad libitum-fed hamsters between 8 and 30 days of food restriction at $P < 0.05$.

Figure 3.13



Mean and standard error of the mean of the number of vaginal marks produced during a test for appetitive sex behavior in ovariectomized females treated with estradiol in Experiment 3.3. Females were food-restricted or fed ad libitum for 30 days prior to the test. Half of each food group, the “Mated” females were conditioned with males whereas the other half, the “Unmated” females were conditioned with an empty compartment. * Food-restricted hamsters significantly different from ad libitum-fed hamsters by $P < 0.05$.

Chapter 4

Food Restriction-induced Changes in Gonadotropin-inhibiting Hormone Cells Are Sssociated With Changes in Sexual Motivation and Food Hoarding, but Not Sexual Performance and Food intake

Metabolic control of the reproductive system has been demonstrated in every order of the class Mammalia (Bronson, 1989). Reproduction is inhibited when the availability of oxidizable fuels is scarce, and reproduction is rapidly stimulated when fuels become abundant (Bronson, 1986;Szymanski et al., 2007). Mechanisms that measure fuel availability and modulate reproductive processes serve to optimize reproductive success in environments where food availability and energy demands fluctuate (Bronson, 1989;Wade and Schneider, 1992;Schneider, 2006). The mechanisms that switch behavioral priorities from ingestive to reproductive behaviors might occur at multiple loci, including effects on behavioral motivation (the internal desire for food or sex), performance (mating and eating) and the hypothalamic-pituitary-gonadal (HPG) system, including the gonadotropin releasing hormone (GnRH) pulse generator, pituitary gonadotropin secretion, and ovarian steroid secretion. Despite action at multiple loci, the majority of research has focused on metabolic challenges that induce anestrus, inhibit gonadotropin secretion, and stimulate food intake (Kalra et al., 1988;I'Anson et al., 1991;McShane et al., 1992;Wade and Schneider, 1992;Foster et al., 1998;Henry et al., 1999;Cunningham, 2004;Schneider, 2004). Food deprivation and other metabolic

challenges inhibit pulsatile GnRH secretion that, in turn, inhibits pituitary luteinizing hormone (LH) secretion, ovarian steroid synthesis and secretion, and ovarian-steroid-dependent copulatory behavior in a wide variety of species, including Syrian hamsters (McClure, 1962;Morin, 1975;Ronnekleiv et al., 1978;Bronson and Marsteller, 1985;Foster and Olster, 1985;Armstrong and Britt, 1987;Bronson, 1988;Sprangers and Piacsek, 1988;Schneider and Wade, 1989;Thomas et al., 1990;Cameron, 1996;Shahab et al., 1997;Temple et al., 2002;Terry et al., 2005;Shahab et al., 2006).

It is likely, however, that energy deficits influence behavioral motivation even before metabolic challenges become so severe that they induce anestrus. Lean Syrian hamsters become anestrus after a 48 h period of food deprivation, whereas pre-fattened Syrian hamsters, which do not become anestrus, show deficits in paracopulatory behaviors. Fattened Syrian hamsters food-deprived for 24-36 h show significantly decreased appetitive sex behaviors, such as decreased vaginal scent marking, and significantly increased appetitive ingestive behaviors, such as food hoarding (Schneider et al., 2007). Appetitive behaviors bring animals in contact with the goal object (mating partners or food), and often occur separated in time from mating and eating (Sherrington, 1906;Craig, 1917;Lorenz, 1950;Johnston, 1974;Johnston, 1977;Lisk, 1983;Everitt, 1990). Syrian hamster appetitive sex behaviors include vaginal scent marking, an estradiol-dependent behavior that occurs with increasing frequency over days 1, 2, and 3 of the 4-day estrous cycle (with day 4 being proestrus) (Johnston, 1977), and the preference for males vs. food (the time spent with the male minus the time spent with food divided by the total time). Consummatory sex behavior is commonly measured in Syrian hamsters

as the incidence of the lordosis reflex, a posture that allows male intromission on day 4 of the estrous cycle and requires physiological concentrations of plasma estradiol and progesterone, tactile flank stimulation, and male olfactory cues (Lisk, 1983). Flank marking is yet another appetitive social behavior (specifically an agonistic behavior) in Syrian hamsters that is higher in dominant individuals, increases with increases in plasma estradiol concentrations, and is inhibited at the time of estrus by the presence of adult male hamsters (Albers and Rawls, 1989;Albers and Rowland, 1989). With regard to ingestive behaviors, food hoarding is an example of appetitive ingestive behavior, whereas food intake is a consummatory behavior in Syrian hamsters (Smith and Ross, 1950;Waddell, 1951).

Consummatory sex and ingestive behavior can be simultaneously stimulated under special circumstances (Kaplan et al., 1992). Appetitive behaviors, however, are often in conflict, and females must choose between engaging in courtship or foraging for food. In nature, females typically have a choice between ingestive and sex behavior, and the decision can impact survival and reproductive success. Thus, we have included appetitive behaviors and the choice between food and males in our experiments on energetic control of ingestive and reproductive behavior in female Syrian hamsters (*Mesocricetus auratus*). By attention to the decisions to engage in either reproductive or ingestive behavior, we hoped to gain insight into hormones and neuropeptides implicated in control of food intake and reproduction, such as gonadotropinin-inhibiting hormone (GnIH), neuropeptide Y (NPY), leptin, insulin, estradiol, and progesterone.

In this experiment, appetitive and consummatory sex and ingestive behaviors were examined over the course of food restriction to test the following hypotheses: 1) Appetitive behaviors are more sensitive than consummatory behaviors to the effects of mild food restriction, 2) changes in appetitive behavior are correlated with increases in neural activation in cells that contain GnIH, and 3) cells that contain NPY project to the vicinity of GnIH cells in the dorsomedial hypothalamus (DMH). GnIH and NPY were examined for the following reasons.

GnIH has been implicated in environmental control of reproduction and food intake. GnIH was first identified from quail hypothalamus. Treatment with the newly identified peptide inhibited gonadotropin release from pituitary cells in vitro in a dose-dependent manner, and hence it was named gonadotropin-inhibiting hormone (Tsutsui et al., 2000). Orthologous neuropeptides were subsequently discovered in a wide range of vertebrate species (reviewed in (Bentley et al., 2010;Kriegsfeld et al., 2010;Smith and Clarke, 2010;Tsutsui et al., 2010). Evidence has accumulated that the mammalian homolog of GnIH, RFamide-related peptide-3 (RFRP-3, Arg-Phe-NH2 in the C terminus), acts as a negative regulator of gonadotropin secretion in all species investigated, including hamsters, mice, rats, cattle, sheep, non human primates, and human beings (Kriegsfeld et al., 2006;Johnson et al., 2007;Clarke et al., 2008;Anderson et al., 2009;Smith and Clarke, 2010). In the past 5 years, the accumulated evidence across many mammalian species has revealed many similarities among mammals and birds in the function of the orthologous peptides, and there is general consensus that

“GnIH” is the appropriate nomenclature for both peptides. It is unlikely, however, that inhibition of gonadotropin secretion is the only function of this peptide.

We hypothesize that GnIH is a modulator of sex and ingestive motivation in Syrian hamsters because intracerebroventricular treatment with GnIH disrupts sex behavior of female white-crowned sparrows and male rats (Bentley et al., 2006;Johnson et al., 2007), and increases food intake in male rats (Johnson et al., 2007), sheep, mice, and monkeys (I. J. Clarke, personal communication). GnIH cells in Syrian hamsters are restricted to the DMH, contain estradiol receptors, and show neural activation in response to increased circulating concentrations of estradiol (Kriegsfeld et al., 2006). If GnIH is important for the effects of mild food restriction on the observed changes in behavior motivation in female hamsters, it would be predicted that increases in ingestive motivation (food hoarding) and decreases in sexual motivation (the preference for males vs. food) would be preceded by increases in neural activation in GnIH-immunoreactive (Ir) cells. Our hypothesis would be refuted if there were no increase in cellular activation in GnIH-Ir cells or if, for example, the activation occurred at 12 days of restriction even though behavior changed at 8 days of restriction, too late to account for changes in behavior. Thus, the present experiments examined cellular activation in GnIH-Ir cells and appetitive sex and ingestive behavior after either 0, 4, 8, or 12 days of 25% food restriction or after 4 or 8 days of ad libitum feeding to females previously food-restricted for 12 days.

NPY is a hormone that has long been studied in relation to energy balance and reproduction, and more recently, NPY has been implicated specifically in appetitive aspects of ingestion. NPY gene expression is increased in discrete nuclei of the hypothalamus, including the DMH, in response to energy restriction in rodents, including Syrian hamsters (Brady et al., 1990;Jones et al., 2004). Intracerebroventricular treatment with NPY rapidly increases food intake and suppresses mating behavior of male and female rodents, including Syrian hamsters (Clark et al., 1985;Stanley and Leibowitz, 1985;Kulkosky et al., 1988;Corp et al., 2001;Jones et al., 2004), and some data are consistent with the idea that appetitive aspects of behavior are more sensitive to NPY than consummatory aspects of behavior (Ammar et al., 2000). Most relevant to the present study, food hoarding is increased by treatment with NPY agonists and decreased by treatment with antagonists to specific NPY receptors in Siberian hamsters (Day et al., 2005). Investigators interested in NPY effects on food hoarding have focused on the paraventricular nucleus (PVH) and arcuate nucleus (Arc) of the hypothalamus and the perifornical area, but not the DMH. Thus, we double-labeled for GnIH-Ir and NPY-Ir to determine whether there are NPY projections to the GnIH cells in the DMH. In addition, we measured plasma levels of progesterone, leptin, insulin, estradiol because they are putative orexigenic agents and anorectic hormones implicated in control of energy balance and reproduction in a number of species including Syrian hamsters (Wade et al., 1991;Ahima et al., 1996;Schneider et al., 1998;Eckel, 2004;Klingerman et al., 2010) that have not been measured at different durations after mild food restriction and re-feeding and in relation to changes in GnIH.

Materials and Methods

All subjects were adult (60-90 days of age), female Syrian hamsters obtained from Charles River Breeding Laboratories (Wilmingon, MA). Upon arrival, hamsters were housed singly in opaque, Nalgene cages (31 × 19 × 18-cm) in a room maintained at 23 ± 1°C with a 14:10 light-dark cycle (lights on at 2200 hours). Hamsters were fed Harlan Rodent Chow 2016 and water was available at all times. All procedures were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, the United States Department of Agriculture, and a protocol approved by the Lehigh University Institutional Animal Care and Use Committee.

Experiment 4.1: Effects of Energy Restriction on Behavior, GnIH, and Circulating Hormones

This experiment was designed to examine cellular activation in GnIH cells and circulating hormones after testing for appetitive and consummatory sex and ingestive behaviors in animals subject to mild food restriction for varying durations.

Preference Apparatus

Hamsters were acclimated, trained and tested in a preference apparatus designed to duplicate aspects of their native habitat, and to allow quantification of behaviors associated with the motivation to engage in either sex or ingestive behavior (Schneider et al., 2007). Hamsters in the wild live in isolation in underground burrows from which

they emerge for only 90 min per day at dawn and dusk, and spend virtually every minute of this time foraging for and hoarding food (Gattermann et al., 2008). Matings have been observed only at the entrance to the female burrow. Together, these considerations suggest that decisions about whether to engage in ingestive or sex behaviors that occur near the burrow entrance during the 90 min above-ground foraging period are relevant to their reproductive success. Thus, each preference apparatus consisted of a home cage for the subject female connected via a vertical tube to two boxes: One with an adult male hamster (male box) and another box containing a food source (food box). Home cages were made from opaque, Nalgene cages (31 × 19 × 18-cm) lined with fine wood shavings with a door that was kept closed when the animals were not being trained or tested. The vertical tube was 134 cm in length and was connected to tubes in a T-configuration that were 40-50 cm in length. The food box contained a weighed amount (150 ± 5 g) of hoardable pellets made from standard laboratory chow (Harlan Rodent Chow 2016) that was broken into 2 cm pieces, a size that permits pouching and enables hamsters with full cheek pouches to fit readily through the tubes. The male boxes for the stimulus hamsters were made from clear, Plexiglas cages (27 × 20 × 15 cm) with wire barriers that allowed auditory, olfactory, and visual interaction, but prevented mating or fighting. The stimulus male boxes did not contain food or water.

Females were acclimated to the home cage for 24 h/day for at least 1 week prior to testing, which reduced any tendencies to sleep, move bedding, or hoard food into any other compartments during later preference testing. After acclimation to the home, females were trained to expect access to the food and male boxes at the onset of the dark

period. Hamsters experienced training sessions with the food source box once a day for 2 days on days 1 and 2 of the estrous cycle, and training sessions with the male box once a day for 2 days on days 3 and 4 of the estrous cycle. Training is described in detail in two previous publications (Klingerman et al., 2010;Klingerman et al., 2011)

Females that showed at least two consecutive estrous cycles and had been acclimated and trained in the preference apparatus were first tested for baseline behaviors including food hoarding, vaginal scent marking, flank marking, and male preference calculated as (time spent with the male – time spent with food) / total time.

During baseline testing, 24 h food intake was measured for at least 4 days prior to the start of the experiment to obtain a 4-day average daily intake. The 25% food-restricted females were given 75% of their baseline daily intake by giving a pre-weighed food ration immediately after behavior testing, approximately 2 hours after the onset of the dark phase of the light-dark cycle. For all food-restricted females, restriction started on day 4 of the estrous cycle, and all females were sacrificed on day 4 of a subsequent cycle. This level of food restriction was chosen because previous experiments showed that 25% food restriction for up to 16 days does not induce anestrus (Klingerman et al., 2010;Klingerman et al., 2011).

After baseline testing, the 48 hamsters were randomly placed into 1 of 6 groups that did not differ significantly in body weight (115-175 g). The groups included hamsters that were food-restricted by 25% (fed 75% of ad libitum food intake determined during baseline) for 4 days (n = 6), food-restricted for 8 days (n = 6), food-restricted for 12 days (n = 12), food-restricted for 12 days and re-fed ad libitum for 4 days (n = 6),

food-restricted for 12 days and re-fed ad libitum for 8 days (n = 6), or fed ad libitum (n = 12).

Testing began at the onset of the dark phase of the photoperiod (1200 h) on day 3 and was conducted under dim, red illumination. The door to the home cage was opened and females were allowed access to both the male and food boxes for a total of 90 min. During the first 15 min, vaginal marking, flank marking, food hoarding and eating as well as location (male, food, or home cage) were recorded. After 15 min of observation, the experimenter stopped recording and the test continued for an additional 75 min (90 min total); i.e., the females continued to have access to both the male and food boxes. After the 90 min test was complete, the hamsters were returned to their respective cages and the doors to the home cages were closed. Weight of food in the home cage and food box was measured and recorded to determine the amount of food hoarded and eaten during the 90 min test.

Blood Collection and Perfusion

Female hamsters were tested in the preference apparatus on day 3 of the estrous cycle, and at the same time the next day, they were euthanized and a terminal blood sample was taken. Plasma was assayed for estradiol and progesterone concentrations to determine effects of food restriction, and to determine whether levels were below those that would induce lordosis. Plasma insulin and leptin concentrations were assayed to determine the effects of chronic restriction. In order to avoid the confounding effects of meals and cephalic phase hormone release, both food-restricted and ad libitum-fed

animals were given access to the amount of food normally fed to the food-restricted females for 15 min 4 hours before blood collection. This schedule was chosen because previous results showed that Syrian hamsters do not show post-fast hyperphagia, and plasma insulin and leptin concentrations are not significantly increased in Syrian hamsters until more than 4 h after a meal (Schneider et al., 2000). Thus, plasma hormone concentrations in our different groups of females would be expected to reflect length of food restriction rather than effects of meals. All hamsters were sacrificed before the onset of the dark phase of the photoperiod (1200 h) by an overdose of sodium pentobarbital (Ovation Pharmaceuticals, Inc., Deerfield, IL). Blood was centrifuged at 3000 rpm and 5°C for 20 min. Plasma was collected and frozen at -20°C until analysis.

Animals were perfused intracardially with phosphate buffered saline (PBS, pH 7.4 at 4°C) followed by 4% paraformaldehyde in PBS at the same temperature. Brains were removed, post-fixed for 24 h at 4°C in 4% paraformaldehyde, and stored at 4°C in 20% sucrose and 0.001% thimerosol until sectioning. All brains were sectioned within 30 days using a freezing microtome set at 40 μm. Hypothalamic brain sections were placed into polyvinyl pyrollidone (PVP) and stored at -20°C until immunohistochemical staining.

Immunohistochemistry

Cellular activation in GnIH-containing cells was measured by double-labeling for intranuclear Fos, the product of the immediate-early-gene, c-fos, a well established marker of changes in cellular activity in response to stimuli in rodents (Hoffman et al.,

1993). Tissue was collected and every 4th 40 μm section was double-labeled using fluorescence immunohistochemistry. Fos (1:50,000; Jackson ImmunoResearch Laboratories, West Grove, PA) was amplified with biotinylated tyramine (0.6%) for 30 min at room temperature prior to incubation in CY-2 conjugated streptavidin (1:200; Jackson ImmunoResearch Laboratories) for 1 h. Following labeling for Fos, sections were labeled using an antibody directed against GnIH specifically for Syrian hamsters (1:10,000; PAC 1365), with CY-3 donkey anti-rabbit (1:200) as the secondary antibody/fluorophore. The antibody has been extensively characterized in this species and has been shown to be specific to RFRP-3 with no cross-reactivity with related RFamide peptides (Gibson et al., 2008).

The population of GnIH-expressing cells is restricted to the DMH in Syrian hamsters, unlike in sheep, birds, and other rodents (Kriegsfeld, 2006;Kriegsfeld et al., 2006;Kriegsfeld et al., 2010). In addition, we typically find a few scattered peri-DMH cells in Syrian hamsters. We used standard procedures for dual-label immunofluorescence to count all double-labeled cells in Syrian hamster sections that contained the DMH (see below under Light and Confocal Microscopy).

The mean number of GnIH cells was obtained by counting the number of cells that were labeled by the GnIH antibody in each animal, taking the sum of all GnIH labeled cells for each experimental group, and dividing by the sample size of the group. The percent of GnIH cells that were also labeled for Fos was calculated for each animal by counting the number of double-labeled cells, dividing by the total number of GnIH cells (the sum of the Fos-labeled plus the nonlabeled cells), and multiplying by 100.

Light microscopy

Brain sections processed for immunocytochemistry were mounted and coverslipped and were investigated using a Zeiss Z1 microscope. Sections were examined using the standard wavelengths for CY-2 (488 nm) and CY-3 (568 nm). Every 4th section through the DMH was assessed, and those areas expressing GnIH-Ir were recorded for coexpression with Fos protein using confocal microscopy (see below). For light microscopy, areas identified as having double-labeled cells were digitally captured at 200x in 8 bit greyscale using a cooled CCD camera (Zeiss). The total number of GnIH cells and the percentage of cells expressing Fos were recorded by two independent observers blind to the experimental conditions.

Confocal microscopy

Cells characterized as double-labeled with Fos/GnIH at the conventional microscopy level were confirmed with confocal microscopy to ensure that Fos was expressed within the cells rather than in overlapping cells in the same field of view. Likewise, cells classified as single-labeled were assessed to ensure that the conventional microscopy strategy did not result in false negatives. At least 10% of those cells quantified using conventional microscopy were assessed in confocal scans for Fos co-labeling. Regions of the brain with putative double-label identified at the light level were scanned at 400 × using confocal microscopy. Cells were observed under a Zeiss Axiovert 100TV fluorescence microscope (Carl Zeiss, Thornwood, NY) with a Zeiss LSM 510 laser scanning confocal attachment. The sections were excited with an Argon-Krypton laser using the standard excitation wavelengths for CY-2 and CY-3. Stacked

images were collected as 1.0 µm multitract optical sections. Using the LSM 3.95 software (Zeiss), red and green images of the sections were superimposed. GnIH cells in the DMH were examined through their entirety in 1.0 µm steps. Each microscope channel (i.e., CY-2 and CY-3) was excited independently in the same focal plane, and the photographs were merged into a single red-green image (because in fluorescence confocal microscopy, two fluorescent channels cannot be viewed simultaneously). The software program, Adobe Photoshop, was used to turn individual channels on (illuminated) and off independently, in order to confirm double-labeling of individual cells. First, we identified cells with a visibly stained nucleus in the GnIH channel, and then, when the other channel was illuminated, noted those in which Fos-staining cells filled the void. This procedure greatly reduced the potential for counting false positives compared to dual-label quantification performed using two chromogens.

To examine NPY contacts, GnIH-Ir cells with putative NPY contacts were scanned though the extent of each cell in 0.5 µm increments. Cells characterized as double-labeled with NPY/GnIH at the conventional microscopy level were confirmed with confocal microscopy to ensure that Fos was expressed within the cells rather than in overlapping cells in the same field of view. Likewise, cells classified as single-labeled were assessed to ensure that the conventional microscopy strategy did not result in false negatives. Only those cells in which the NPY- labeled fiber contacted a GnIH-Ir cell in the same 0.5 µm scan were counted as close contacts.

Leptin and Insulin Radioimmunoassay

Blood plasma was analyzed for leptin using the Multi-Species Leptin Radioimmunoassay (RIA) kit (Millipore, St. Charles, MO). Samples were run in duplicate in the same assay with assay limits between 1.0 ng/ml and 50 ng/ml. Similarly, plasma insulin was measured in duplicate using a Rat Insulin RIA kit (Millipore, St. Charles, MO) adjusted to use 50 µl of plasma with assay limits between 0.01 ng/ml and 10.0 ng/ml. Insulin and leptin assays were performed by Millipore Biomarker Services (St. Charles, MO).

Estradiol and Progesterone Radioimmunoassay

Blood plasma was analyzed for estradiol and progesterone using RIAs (TKE21 and TKPG2, Siemens Medical Solutions Diagnostics, Los Angeles, CA). Assay limits were between 10.0 pg/ml and 1035.4 pg/ml for the estradiol assay and 0.09 ng/ml and 13.0 ng/ml for the progesterone assay. For progesterone values to fall within the acceptable range, blood plasma was diluted 1:10 prior to analysis. Estradiol and progesterone assays were conducted by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (Charlottesville, VA).

Statistical Analysis

Behavioral, hormonal, and immunohistochemical data were analyzed using one-way analysis of variance (ANOVA) with different durations of food restriction as the main effect. In order to meet the assumption of homogeneity of variances, some behavioral scores were log $(x + 1)$ transformed prior to the ANOVA (Sokal and Rohlf,

1969). This applied to food hoarding, male preference, and flank marking. $Fmax_{(5, 4)}$ for those variables after log transformation were 4.8, 11.72 and 12.9 respectively, showing that there were no significant differences among the variances of the transformed scores. Means and standard errors of the means of the raw, untransformed scores appear in all figures for ease of presentation. When main effects were significant, post hoc comparisons were made using Duncan's Multiple Range test. Correlation coefficients were calculated to determine whether there was a significant association between cellular activation in GnIH cells and each behavior variable, or between plasma hormone concentrations and each behavioral variable. Differences were considered statistically significant if $P < 0.05$.

Experiment 4.2: Effects of Food Deprivation and Body Fat Content on Cellular Activation in GnIH Cells and NPY Projections to the DMH

These two experiments examined cellular activation in GnIH cells in the DMH that were either susceptible to or buffered from severe metabolic challenges (food deprivation). Previous work determined that adult, estrous-cycling hamsters below 120 g in body weight were highly likely to show anestrus after 48 h or more of food deprivation, whereas those above 125 g were buffered from the effects of food deprivation due to their higher body fat content and the ability to oxidize free fatty acids from lipids stored in adipose tissue (Schneider and Wade, 1989).

Hamsters that were the same age, with the same diet composition, were created by feeding diets that differed in the energy required to ingest them. The low body weight group was fed 4 pellets (approximately 20 g) of standard rodent chow in the wire hopper that hangs into the ceiling of the cage. The high body weight group was fed powdered rodent chow ad libitum on the floor of the cage. The former group showed a high level of activity as they stood upright and gnawed at the pellets. The latter group, those fed the powdered chow, expended comparably less energy and gained body weight faster because they were not required to chew their food in order to consume it, and they slept in close proximity, if not right in the food.

In the first experiment, Experiment 4.2A, hamsters were either high (n = 5, 133.13 $\pm$ 2.9) or low body weight (n = 6, 113.6 $\pm$ 3.5) and half of each group was fed ad libitum or food-deprived for 72 h ending on day 4 of the estrous cycle, the day of the LH surge and ovulation. This experiment was designed to determine whether cellular activation in GnIH cells on the day of the LH surge would be affected by the severe energetic challenge known to induce anestrus, and whether having a high body fat content prior to deprivation would buffer this effect. LH assays were performed by The University of Virginia Ligand Assay and Analysis Core Laboratory Services using the Rat Sandwich-IRMA assay. Two-way ANOVA, with food availability and prior body weight as the two main factors, was used to analyze the data.

The second experiment, Experiment 4.2B, was designed to examine cellular activation in GnIH cells earlier, in the follicular phase, during the initiation of effects of food restriction on the GnRH pulse generator. Thus, 18 hamsters of a high (n = 9, 121.2

$\pm$ 2) or low body weight (n = 9, 104.2 $\pm$ 3.1) were food deprived for either 36 h (euthanized on day 2 of the estrous cycle) or 50 hours (euthanized on day 3 of the cycle). An additional group (n = 6, 131.4 + 2.5) served as ad libitum-fed controls and data were analyzed with a one-way ANOVA.

In both experiments, the blood was sampled and assayed for LH, and hamsters perfused as described for Experiment 4.1. Animals were perfused intracardially with phosphate buffered saline (PBS, pH 7.4 at 4°C) followed by 4% paraformaldehyde in PBS at the same temperature. Brains were removed, post-fixed for 24 h at 4°C in 4% paraformaldehyde, and stored at 4°C in 20% sucrose and 0.001% thimerosol until sectioning. All brains were sectioned within 30 days using a freezing microtome set at 40 µm. Hypothalamic brain sections were placed into PVP and stored at -20°C until staining.

Double-labeling for Fos and GnIH and double-labeling for NPY and GnIH was carried out as described in Experiment 4.1 on every fourth section. NPY fibers were immunostained using an NPY antibody, rabbit polyclonal anti-NPY (1:10,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and was amplified with biotinylated tyramine (0.6%) for 30 min at room temperature prior to incubation in CY-2 conjugated streptavidin (1:200; Jackson ImmunoResearch Laboratories) for 1 h. Following labeling for NPY, sections were labeled using an antibody directed against GnIH specifically for Syrian hamsters (1:10,000; PAC 1365), with CY-3 donkey anti-rabbit (1:200) as the secondary antibody/fluorophore.

Experiment 4.3: Effects of Systemic Injections of GnIH on Appetitive and Consummatory Sex and Ingestive Behavior

16 female Syrian hamsters (8 mo, 144.12 ± 4.98 g) were obtained from an animal colony at Cornell University (Ithaca, NY). Upon arrival, females were housed at 22 ± 1 °C with a 14:10 light-dark cycle (lights on at 2200 h) and trained to our preference apparatus as described previously. After baseline testing, females were randomly grouped by body weight into treatment groups; GnIH or the vehicle, saline. 30 min before testing on day 3 of the estrous cycle, females were given an intraperitoneal injection of 600 ng of GnIH or saline in a similar volume. This dose regimen has been shown to suppress luteinizing hormone secretion in female Syrian hamsters (Kriegsfeld et al., 2006). Testing was conducted similar to baseline and 24 h food intake was also measured. The next day, day 4 of the estrous cycle, females were injected with a similar dose of GnIH or saline intraperitoneally 4 h before the dark phase of the light:dark cycle and sacrificed by a terminal blood collection 30 min later to determine if GnIH is suppressing LH. Briefly, hamsters were anesthetized with isoflurane and blood was collected via cardiac puncture. Blood plasma was collected after centrifugation at 3000 rpm for 15 min. This time of day corresponds to maximal LH in this species (Gibson et al., 2008).

Luteinizing Hormone

Blood plasma was analyzed for LH using the rat LH sandwich assay with monoclonal antibodies against bovine LH and the human LH-beta subunit (sensitivity of

0.07 ng.ml). The assay was performed by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (Charlottesville, VA).

Results

Experiment 4.1: Different Durations of Food Restriction, GnIH and Behavior

Ingestive Behaviors

One-way ANOVA showed a significant main effect of food availability on the amount of food hoarded ($F(5,42) = 2.64$, $P < 0.04$) as well as on the log $(x + 1)$ transform of the amount of food hoarded ($F(5,42) = 5.42$, $P < 0.02$) (Fig. 4.1, top). Similarly, when the amount of food hoarded post-restriction was subtracted from baseline food hoarded, there was a significant effect of food restriction on the change in the amount of food hoarded ($F(5,42) = 2.75$, $P < 0.03$). Post hoc tests showed that the amount of food hoarded was significantly higher in the 8-day and 12-day food restricted groups compared to the ad libitum-fed group and the 4-day food-restricted group ($P < 0.05$).

There was a significant main effect of food availability on the amount of time spent eating during the preference test ($F(5,42) = 7.56$, $P < 0.0001$) (Table 4.1). Hamsters spent significantly more time eating after 4, 8, and 12 days of food restriction compared to hamsters fed ad libitum ($P < 0.05$) (Table 4.1).

The amount of food eaten (g) during the 90-min test (Fig. 4.2C) did not differ significantly among groups fed ad libitum or food-restricted for varying durations.

Reproductive Behaviors

The effect of food availability on the number of vaginal scent marks per 15 min was significant ($F(5,42) = 4.66$, $P < 0.002$) (Fig. 4.1, bottom). Hamsters food restricted for 8 and 12 days showed significantly fewer vaginal scent marks than those fed ad libitum and those food restricted for only 4 days ($P < 0.05$), but those re-fed for 4 and 8 days still showed significantly fewer vaginal scent marks than those fed ad libitum ($P < 0.05$).

The effect of food availability on the number of flank marks was significant ($F(5,42) = 3.48$, $P < 0.01$) as was the log $(x + 1)$ transform of flank marking ($F(5,42) = 4.7$, $P < 0.002$). The number of flank marks in 4-day food-restricted females was significantly higher than that of females fed ad libitum ($P < 0.05$), but the flank marking scores of hamsters in the other groups were not significantly higher than those of hamsters fed ad libitum (Table 4.1).

Male preference was calculated as (the amount of time females spent with a male – the amount of time spent with food) / the total time in the preference apparatus (Table 4.1). There was a main effect of food restriction on male preference ($F(5,40) = 3.81$; $P < 0.007$). There was a main effect of food restriction on the log $(x + 1)$ transform of male preference ($F(5,40) = 3.44$; $P < 0.01$). Post hoc analysis showed that 12-day restricted hamsters had significantly lower male preference than ad libitum-fed female hamsters.

Body Weight

When the hamsters' final body weights were subtracted from initial body weights, the groups differed significantly in the amount of body weight lost ($F(5,42) = 30.92$, $P < 0.0001$) (Fig. 4.2A).

Body weights among the groups were not significantly different at the start of the experiment. The effect of duration of food restriction on final body weight was significant ($F(5,42) = 4.37$, $P < 0.003$) (Fig. 4.2B). Body weights were significantly decreased starting at 4 days after the start of food restriction ($P < 0.05$) compared to hamsters fed ad libitum. Hamsters fed ad libitum throughout the experiment were significantly heavier compared to all other groups except hamsters food-restricted for 12 days and re-fed ad libitum for 8 days (Fig. 4.2B).

<u>GnIH immunoreactivity and cellular activation</u>

Cellular activation in GnIH-Ir cells was calculated as (the number of cells double-labeled for Fos-Ir and GnIH-Ir/ the total number of GnIH-Ir cells) * 100. There was a significant main effect of food availability on cellular activation in GnIH-Ir cells ($F(5,38) = 3.47$, $P < 0.01$) (Fig. 4.3A). Post hoc analysis revealed a significant increase in cellular activation in GnIH-Ir cells at 8 and 12 days of food restriction compared to hamsters fed ad libitum ($P < 0.05$).

There was a significant effect of food restriction on the total number of GnIH-Ir cells ($F(5,38) = 2.88$, $P < 0.03$), with a significant decrease in the number of GnIH cells that were immunoreactive in the females food restricted for 8 and 12 days compared to those fed ad libitum and those food restricted for 4 days ($P < 0.05$) (Fig. 4.3B).

There was a significant main effect of food restriction on the absolute number of double-labeled Fos-Ir/GnIH-Ir cells ($F(5,38) = 2.457$, $P < 0.05$). Post hoc analysis showed significant increases in the number of double-labeled cells 4 and 8 days after restriction ($P < 0.05$) (Fig. 3C).

Plasma Leptin, Insulin, Estradiol, and Progesterone Concentrations

There was a significant main effect of food treatment on plasma leptin concentrations ($F(5,41) = 2.50$, $P < 0.05$) (Fig. 4.4). Post hoc comparisons revealed that plasma leptin concentrations did not differ between ad libitum-fed and food-restricted females after any level of food restriction. However, females food-restricted for 12 days and re-fed ad libitum for 4 days had significantly higher plasma leptin concentrations compared to females fed ad libitum. The effect of food restriction or re-feeding on plasma insulin, progesterone, or estradiol concentrations were not significant (Fig 4.4).

Correlations

Changes in food hoarding during food restriction (Fig. 4.1, top) showed a striking resemblance to cellular activation in GnIH-Ir cells (Fig. 4.3A). There was a significant positive correlation between the amount of food hoarded and percent of GnIH-Ir cells that were positive for Fos-Ir ($r = 0.585$; $P < 0.0001$), and a significant negative correlation between food hoarded and GnIH-Ir cell count ($r = 0.436$; $P < 0.003$). The amount of food hoarded was significantly correlated with body weight loss ($r = 0.368$; $P < 0.01$), but not with raw body weight. The correlations between food hoarding and other

variables were not statistically significant (body weight, leptin, insulin, estradiol, or progesterone concentrations).

There was a significant negative correlation between time spent eating and body weight ($r = 0.437$; $P < 0.002$). The correlations between time spent eating and other variables were not statistically significant (change in body weight, number of GnIH-Ir cells, and percent of GnIH-Ir cells that were positive for Fos-Ir, plasma insulin, leptin, estradiol, and progesterone concentrations).

The correlations among 90-min food intake and the other variables were not statistically significant (body weight, change in body weight, number of GnIH-Ir cells, or percent of GnIH-Ir cells that were positive for Fos-Ir, plasma insulin, estradiol, and progesterone concentrations).

There was a significant negative correlation between vaginal scent marks and cellular activation in GnIH-Ir cells ($r = -0.314$; $P < 0.04$) and a positive correlation between the number of vaginal scent marks and the number of cells that showed GnIH-Ir ($r = 0.365$; $P < 0.02$). Vaginal scent marks were significantly negatively correlated with body weight loss; the more body weight lost, the fewer vaginal scent marks ($r = -0.619$; $P < 0.0001$), but vaginal scent marks were not significantly correlated with final body weight. Vaginal scent marks were also positively correlated with plasma progesterone concentrations ($r = 0.354$, $P < 0.02$), but not with leptin, insulin, or estradiol concentrations.

There were no significant correlations between flank marks and any other variables (number of GnIH-Ir cells, cellular activation in GnIH-Ir cells, change in body weight, insulin, estradiol, or progesterone concentrations).

There was a significant negative correlation between male preference and change in body weight ($r = 0.352$; $P < 0.01$), but the correlations between male preference and other variables were not statistically significant (body weight, number of GnIH-Ir cells, cellular activation in GnIH-Ir cells, plasma leptin, insulin, estradiol, or progesterone concentrations).

There was a significant positive correlation between body weight and plasma progesterone concentrations ($r = 0.302$; $P < 0.04$) and between body weight and plasma leptin concentrations ($r = 0.285$; $P < 0.05$), but not between body weight and plasma insulin or estradiol concentrations. Body weight was not significantly correlated with either the number of GnIH cells, or the percent of GnIH-Ir cells that were positive for Fos. Change in body weight was significantly positively correlated with plasma progesterone concentrations ($r = 0.451$; $P < 0.001$) and the number of GnIH-Ir cells ($r = 0.459$; $P < 0.002$) and significantly negatively correlated with the percent of GnIH-Ir cells that were positive for Fos ($r = 0.570$; $P < 0.0001$).

<u>Experiment 4.2: Effect of Metabolic Challenges on NPY Fibers in the DMH</u>

In Experiment 4.2A, females with either high or low body weight were either fed ad libitum or food deprived for 72 h from day 1 to day 4 of the estrous cycle (Fig. 4.5). Previous work showed that the lean, food-deprived females would become anestrus,

whereas those that were fat at the start of deprivation would be buffered from the effects of deprivation (Schneider and Wade, 1989; 1990). Two-way ANOVA showed a significant main effect of food deprivation on final body weight, with lean females weighing less than fat ($F(1,6) = 9.78$, $P < 0.02$). Post hoc analysis showed that food-deprived lean females weighed significantly less than food-deprived, fat females ($P < 0.05$). The same analysis of the change in body weight over the course of food deprivation showed a significant main effect of food deprivation ($F(1,6) = 49.51$, $P < 0.0004$). Two-way ANOVA showed a significant main effect of food deprivation on the percent of GnIH-Ir cells that were positive for Fos-Ir ($F(1,6) = 7.69$, $P < 0.03$), no significant effect of body weight group, and no significant interaction. The more body weight lost, the higher the increase in percent of GnIH-Ir cells that were positive for Fos-Ir, and this correlation was significant ($r = 0.72$, $P < 0.02$). Body weight loss was significantly negatively correlated with the number of cells that were immunoreactive for GnIH ($r = 0.72$, $P < 0.02$). Neither the percent of GnIH-Ir cells positive for Fos-Ir nor the number of GnIH-Ir cells was significantly correlated with final body weight. There was a significant main effect of food deprivation or ad libitum feeding on plasma LH concentrations ($F(2,4)= 16.217$, $P < 0.01$). As expected, the lean, food-deprived females had plasma LH concentrations significantly lower (0.04 ± 0.0001 ng/ml) than the fat, food-deprived (0.131 ± 0.2 ng/ml) and the ad libitum-fed control females (fat and lean combined 0.205 ± 0.038 ng/ml).

In Experiment 4.2B, females were sacrificed after either 1.5 or 2.5 days of food deprivation during the follicular phase of the estrous cycle (Days 1 and 2, the follicular

phase) to determine whether there were changes in GnIH that occur in the early stages of metabolic challenge that would be expected to inhibit the GnRH pulse generator in lean, but not fat females (Morin, 1986). One-way ANOVA showed no significant effect of treatment group on the percent of GnIH-Ir cells that were positive for Fos-Ir, and a significant effect of treatment group on the number of GnIH-Ir cells ($F(2,16) = 27.95$, $P < 0.0001$) (Table 4.2). Both food-deprived groups (30 and 50 h of deprivation) had significantly fewer GnIH-Ir cells than did the ad libitum-fed controls ($P < 0.0001$). The percent of GnIH-Ir cells that were positive for Fos-Ir increased linearly with the amount of body weight loss and this correlation was significant ($r = 0.62$, $P < 0.004$). This variable was also significantly positively correlated with final body weight ($r = .576$, $P < 0.01$). The number of GnIH-Ir cells was also significantly negatively correlated with the amount of body weight lost ($r = -0.58$, $P < 0.01$) and with final body weight ($r = -0.59$, $P < 0.01$).

Double-labeling for GnIH-Ir and NPY-Ir revealed that NPY-Ir nerve fibers were densely packed in the DMH, and that putative NPY terminals can be observed in close proximity to GnIH cell bodies within this brain area (Fig. 4.7) at low power light microscopy and confirmed at high power light microscopy and confocal microscopy. An average of 41.46% GnIH-Ir cell bodies per animal ($n = 6$) receive contacts from NPY-Ir fibers in the DMH.

<u>Experiment 4.3: Effects of Systemic Injections of GnIH on Appetitive and Consummatory Sex and Ingestive Behavior</u>

Ingestive Behaviors

There was no main effect of systemic, exogenous GnIH on 90-min food hoarding or intake (Fig. 4.8). We also subtracted post-injection food hoarding and intake from baseline and found no significant effect of treatment. There was also no main effect of GnIH on 24-h food intake or time spent eating or hoarding during the 15 min observation period (data not shown).

Reproductive Behaviors

There was no main effect of exogenous GnIH on male preference calculated as (time spent with a male – time spent with food) / total time (Fig. 4.9). There was also no effect of GnIH on male preference when we subtracted post-injection values from baseline. There was also no main effect of exogenous GnIH on vaginal marking (Fig 4.9). However, when we subtracted the number of vaginal marks post-injection from baseline, there was a significant decrease in vaginal marking after GnIH treatment ($F(1,14) = 15.02$, $P < 0.002$).

Body Weight

Groups of hamsters did not differ in body weights at the start of the experiment. Likewise, one injection of GnIH had no significant effect on body weight the next day (145.6 ± 6.8 & 145.7 ± 6.6 g respectively).

Luteinizing Hormone

Intraperitoneal GnIH (600 ng given 4 h before the dark phase of the light:dark cycle) did not suppress the luteinizing hormone surge compared to females given vehicle (22.2 ± 1.2 & 21.8 ± 1.3 ng/ml respectively).

Discussion

The primary findings were 1) a linear effect of energy availability on ingestive and sex behavior in Syrian hamsters, with appetitive behaviors most sensitive and ovarian steroid secretion the least sensitive to food restriction or deprivation (Figs. 4.1, 4.4), 2) a linear effect of energy availability on cellular activation in GnIH in the DMH (Fig. 4.3) significantly correlated with food hoarding and negatively correlated with vaginal scent marking, 3) no significant effect of food restriction on plasma leptin, insulin, estradiol, or progesterone concentrations (Fig. 4.4) and no significant correlation among hormone concentrations and cellular activation of GnIH immunoreactive cells, and 4) marked projections of NPY immunoreactive fibers in close apposition to GnIH-containing cell bodies in the DMH (Fig. 4.7). Together, these results are consistent with the idea that a wide range of metabolic deficits have effects on the GnIH system that are significantly correlated with changes in motivation. These effects on cellular activation in GnIH-Ir cells occurred in females that showed normal circulating levels of ovarian steroids, i.e., females that were not hypogonadotropic. Furthermore, projections from NPY-Ir cells to GnIH cells in the DMH are one possible route of transmission of information about energy availability to the GnIH system.

By using mild levels of food restriction at different durations, we were able to dissociate appetitive from consummatory behaviors. Appetitive, but not consummatory behaviors were affected by shorter durations of food restriction. This, in turn, enabled the investigation of neuroanatomical correlates. Food hoarding and vaginal scent marking were significantly affected at 8 and 12 days of 25% food restriction, even though there was no effect on the consummatory behavior, food intake (Figs. 4.1 and 4.2). Mild food restriction did not lead to significant decreases in circulating levels of estradiol and progesterone (Fig. 4.4A and B), confirming that estrous cycles were not disrupted. The body weight, age and level of food restriction were chosen for these experiments because two previously published experiments showed that, under these conditions, estrous cycles were not disrupted and females showed no deficits in lordosis frequency or duration (Klingerman et al., 2010;Klingerman et al., 2011). Food restriction affected paracopulatory behaviors, such as vaginal scent marking, but these effects differed from those on flank marking, as would be expected if effects on sexual motivation are at least partially separable from effects on agonistic or other social behaviors.

The most striking and unexpected observation was the close correlation between cellular activation in GnIH-Ir cells and the change in appetitive behaviors seen in mildly food-restricted females in Experiment 4.1 (Fig. 4.3). Just as the appetitive behaviors (food hoarding and vaginal scent marking) showed significant changes at 8 and 12 days after restriction, cellular activation in GnIH-Ir cells also increased at these same time points. Restoration of food hoarding and Fos/GnIH-Ir to baseline levels occurred at the same time after re-feeding (Figs. 4.1 and 4.3). In contrast to vaginal scent marking, flank

marking was not significantly correlated with changes in cellular activation in GnIH-Ir cells or number of GnIH-Ir cells. Thus, sexual motivation and agonistic motivation are at least partially separable. Changes in cellular activation in GnIH-Ir cells in nonhypogonadotropic Syrian hamsters suggests that this peptide plays a role in control of behavioral motivation, rather than or in addition to control of the HPG system.

An unexpected finding was that in Experiment 4.2A, lean and fat food-deprived females differed significantly in their body weight and plasma LH concentrations, but did not differ in cellular activation in GnIH-Ir cells (Fig. 4.5A and 4.6B). Lean, but not fat, food-deprived females were expected to become anestrus based on previously published data (Schneider and Wade, 1989; 1990). Consistent with this prediction, the lean, food-deprived females had lower mean LH levels than fat, food-deprived females, yet both groups showed significant increases in cellular activation in GnIH-Ir (Fig. 4.6B). Thus, it is possible that in Syrian hamsters, GnIH has important effects on behavioral motivation without inhibition of LH secretion.

Food restriction significantly increased the number of Fos-positive GnIH-Ir cells as well as the percent of the total GnIH-Ir cells that were Fos-positive. Food restriction did not significantly increase the number of GnIH-Ir cells, and, in some groups, decreased the number GnIH-Ir cells (Fig. 4.3B and 4.6A). The increase in the number of activated GnIH-Ir cells coupled with a decreased number of GnIH-Ir cells might reflect increased GnIH release without a compensatory increase in GnIH synthesis. There is precendence in the literature for a decrease in cell number concomitant with an increase in cellular activation and release of peptide. For example, gonadatropin releasing-

hormone (GnRH)-Ir cells in rats also decrease in number during the latter part of the LH surge when the number and percent of Fos-Ir/GnRH-Ir double-labeled cells increases (Lee et al., 1990). Perhaps if we had sacrificed the hamsters earlier, the GnIH-Ir cell population might have remained stable in number. This could be confirmed by examination of GnIH-Ir at earlier time points, by examination of GnIH-Ir in hamsters treated with agents that block axon transport, and by measuring GnIH gene expression using in situ hybridization.

Another possibility is that increased cellular activation along with the observed decrease in the total number of GnIH-Ir cells represents inhibition of GnIH synthesis and secretion. This is unlikely because the decrease in cell number in this experiment did not occur in all experimental groups (for example the 4-day food-restricted group) (Fig. 4.3B), and there was a significant increase in double-labeled cells in all food-restricted females (Fig. 4.3C) (not just an increase in the percent of cells). However, a different environmental factor, short day length, also causes a decrease in GnIH-Ir cell number along with gonadal regression and inhibition of LH secretion (Kriegsfeld et al., 2010).

The decrease in GnIH-Ir cell number, along with an increase in the percent of GnIH cells that were activation closely correlated with behavioral motivation might be a reflection of a subpopulation of cells that is particularly responsive to energy availability. Evidence in other species suggests that KiSS-1 and GnIH act together to coordinate the effects of day length and food availability. KiSS-1 expression increases with food restriction in Siberian hamsters (Paul et al., 2009), is associated with increased GnRH and LH secretion, and has been located in the DMH of rats (Brailoiu et al., 2005), although it

is not known whether there are KiSS-1-containing cells in the Syrian hamster DMH or sites that project to the DMH. Furthermore, GnIH and Kiss-1 might be implicated in both circadian and seasonal rhythms related to energy balance and reproduction in hamsters. For example, DMH lesions block the effects of short day length on the HPG system in Syrian hamsters. The suprachiasmatic nucleus (SCN) projects to a large proportion of GnIH cells in the DMH, and these project to the vicinity of GnRH cells. Thus, it is plausible that information from peripheral or central oscillators are influenced by metabolic fuel availability and project to the SCN, which, in turn, might influence GnIH, KiSS-1 or other cells in the DMH. Other investigators have suggested that the DMH itself receives information generated by the ingestion of meals in mice and rats (Fuller et al., 2008; Gooley et al., 2006), although other evidence contradicts the idea that the DMH is necessary for meal entrainment of circadian rhythms in rats and mice (Landry et al., 2007; Landry et al., 2006; Moriya et al., 2009).

Food restriction in Experiment 4.1 and food deprivation in Experiment 4.2A had significant effects on both cellular activation and on number of GnIH-Ir cells, but it is not clear how this information about food availability reaches the DMH. Food restriction, for example, had significant effects on appetitive behaviors without significant effect on plasma concentrations of ovarian steroids, insulin, or leptin, suggesting that information about fuel availability reaches GnIH cells via other means, e.g., via changes in plasma ghrelin or direct information about the availability of oxidizable metabolic fuels detected in periphery, brain stem or hypothalamic areas that project to GnIH cells.

One possibility is that GnIH cells in food-restricted females are more responsive to estradiol than those GnIH cells in females fed ad libitum. As expected, plasma estradiol concentrations did not differ significantly among the groups food restricted for different durations (Fig. 4.4). Thus, one possible explanation is up-regulation of estradiol receptors (ER) on GnIH cells. A similar suggestion has been made regarding up-regulation of ER in other brain areas involved ingestive behavior and a down-regulation of ER in brain areas involved in lordosis (Li et al., 1994;Panicker et al., 1998). The DMH was not examined in these latter studies. However, ER-α co-localizes with GnIH cells in the Syrian hamsters DMH, and these cells respond to estradiol stimulation with significant increases in cellular activation (Kriegsfeld et al., 2006). Thus, future experiments will determine whether different levels of food restriction (mild to severe) down-regulates ER-α in GnIH cells in the DMH, or whether the effects of food restriction might occur downstream or independent from ER-α-containing GnIH cells.

One such downstream mediator might be GnRH. GnRH and its metabolites have well-documented facilitory effects on sex behavior that are unrelated to LH secretion (Moss and McCann, 1975;Moss and Foreman, 1976;Dudley et al., 1981;Dudley and Moss, 1988;Moss and Dudley, 1990;Dudley and Moss, 1991;Wu et al., 2006). It is plausible that GnIH-mediated inhibition of GnRH secretion accounts for inhibition of appetitive sex behavior in Syrian hamsters. Furthermore, the appetitive ingestive behavior, food hoarding, was significantly increased in the present experiment, consistent with mounting evidence that ingestive behaviors are increased by GnIH (Tachibana et al., 2005;Johnson et al., 2007). GnIH inhibits GnRH secretion in Syrian hamsters

(Kriegsfeld et al., 2006), and at least one form of GnRH (GnRH-II) is inhibitory for ingestive behavior (Kauffman, 2004;Kauffman and Rissman, 2004b;a;Kauffman et al., 2005).

A large body of research implicates NPY in metabolic control of reproduction and ingestive behavior. NPY is a potent orexigenic peptide (Clark et al., 1984;Kulkosky et al., 1988;Corp et al., 2001;Clarke et al., 2005), inhibits sex behavior (Clark et al., 1985;Thornton et al., 1996), and inhibits LH secretion in the presence of low circulating levels of estradiol (Khorram et al., 1987;Sahu et al., 1987;Malven et al., 1992). NPY has greater effects on appetitive than consummatory behaviors (Ammar et al., 2000;Day et al., 2005;Keen-Rhinehart and Bartness, 2007). Furthermore, NPY cell bodies in the DMH and other brain areas have long been implicated in control of energy intake. NPY mRNA is over-expressed in the DMH during the hyperphagia of lactation (Smith, 1993) and in various models of obesity (Kesterson et al., 1997;Guan et al., 1998a;Guan et al., 1998b;Tritos et al., 1998). Increases in NPY gene expression in the DMH of lean rats increases food intake and body weight, and accelerates the development of high-fat diet-induced obesity, and decreased NPY expression in the DMH prevents the hyperphagia, obesity and diabetes of Otsuka Long-Evans Tokushima Fatty rats (Yang et al., 2009). Thus, we were compelled to examine the proximity of NPY projections to GnIH cells in the DMH. Food-deprived females were used to maximize identification of NPY cells. NPY terminals showed strong projections to the DMH and were seen in close apposition to GnIH cells (Fig. 4.7). It is possible that these NPY cells originate in the arcuate nucleus of the hypothalamus, the brain stem, or from within the DMH, all areas where

NPY gene expression has been identified and from which NPY cells project to the DMH in other rodents (Bai et al., 1985;Sahu et al., 1988;Bi et al., 2003).

In summary, these results show a clear correlation between cellular activation in GnIH-Ir cells and appetitive sex and ingestive behaviors. It is not known, however, whether GnIH secretion causes changes in motivation. Results from Experiment 4.3 were inconclusive since GnIH failed to suppress the LH surge. GnIH might be a causal factor for increased hunger and food hoarding, decreased sexual motivation, or both, but it might be a nonfunctional correlate of other causal factors (metabolic events, other hormones, and other neuropeptides such as kisspeptin, NPY, alpha-melanocyte stimulating hormone or orexin). GnRH, for example, might influence ingestive and sex behavior by virtue of its direct link to metabolic cues, since recent evidence shows that GnRH neurons receive dendritic input from outside the blood-brain barrier (Herde et al., 2011). Further work is necessary to determine whether changes in GnIH cells are a fortuitous correlate or a causal factor in control of behavior. Nevertheless, these results are consistent with the idea that GnIH in the DMH, and possibly NPY cells that project to the DMH are part of a system that prioritizes sex and ingestive behavior in order to optimize reproductive success in environments where energy availability fluctuates.

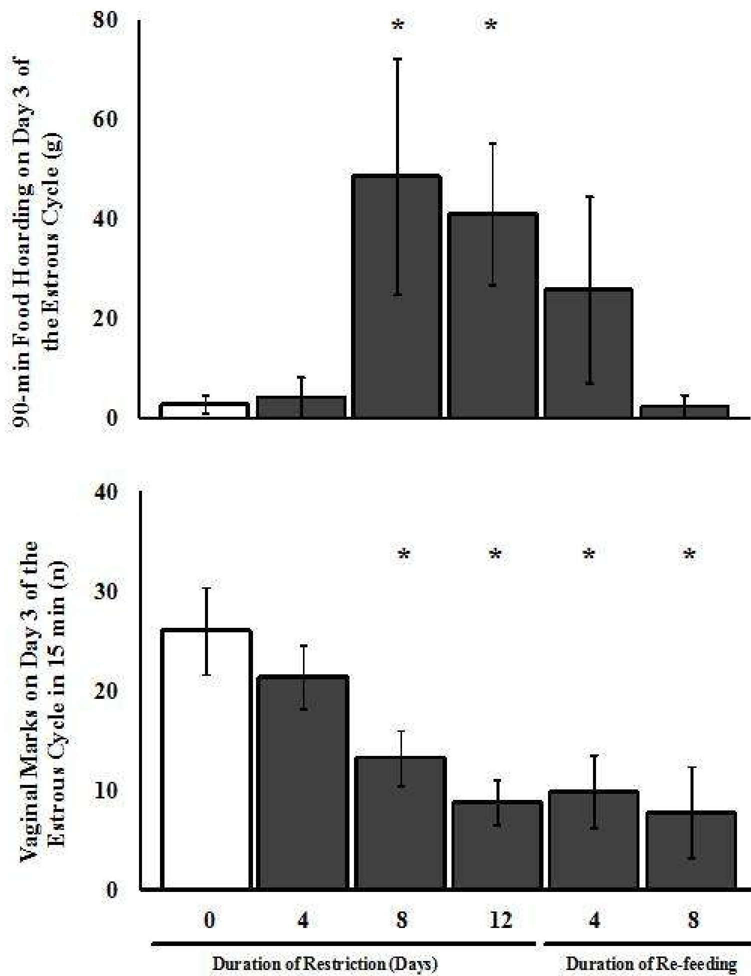
Table 4.1

	Duration of Food Restriction (Days)				Duration of Re-feeding	
	0 (Ad libitum)	4	8	12	4	8
Time Spent Eating (sec)	24.6 ± 6.9	226.7 ± 59.4*	225.8 ± 57.6*	263.8 ± 48.7*	79.2 ± 36.2	36.7 ± 20.9
Time Spent Hoarding (sec)	0 ± 0	0 ± 0	35.0 ± 35.0	12.5 ± 6.2	72.5 ± 64.8	20.8 ± 20.8
Flank Marks (n)	2.4 ± 1.5	7.5 ± 2.1*	2.5 ± 1.3	0.3 ± 0.3	0 ± 0	0.2 ± 0.2
Male Preference (Time with male-time with food)/ Total time	0.31 ± 0.10	0.23 ± 0.13	0.04 ± 0.16	-0.08 ± 0.10	-0.12 ± 0.10	0.22 ± 0.21

Table 4.2

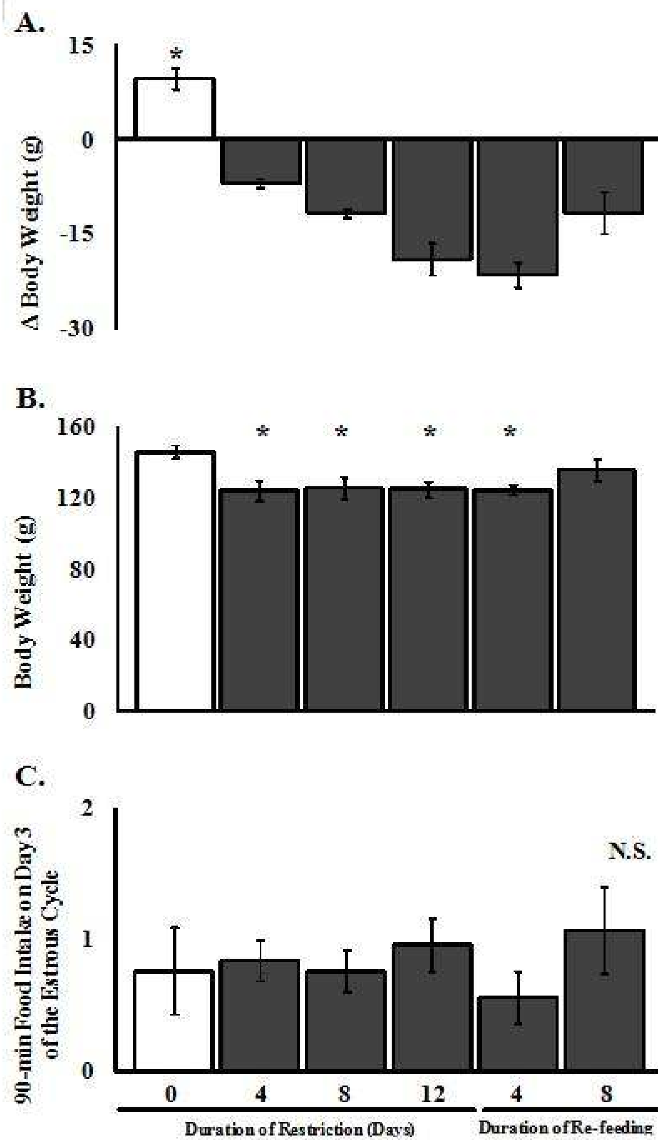
		36 h	50 h	36 h	50 h
	Ad libitum	Fat, Food-deprived		Lean, Food-deprived	
Body weight (g)	131.4 ± 2.5	115.6 ± 3.0*	104.7 ± 7.2*	96.7 ± 0.5*	85.9 ± 2.0*
Δ Body weight (g)	-0.03 ± 0.5	-5.6 ± 0.6*	-12.2 ± 1.0*	-5.4 ± 0.2*	-14.6 ± 1.5*
%FOS+GnIH	24.5 ± 3.3	32.3 ± 6.7	34.3 ± 4.2	35.3 ± 3.8	48.6 ± 8.9
GnIH-ir	122.6 ± 9.9	24.1 ± 5.1*	38.0 ± 5.2*	48.7 ± 11.6*	36.8 ± 15.3*

Figure 4.1



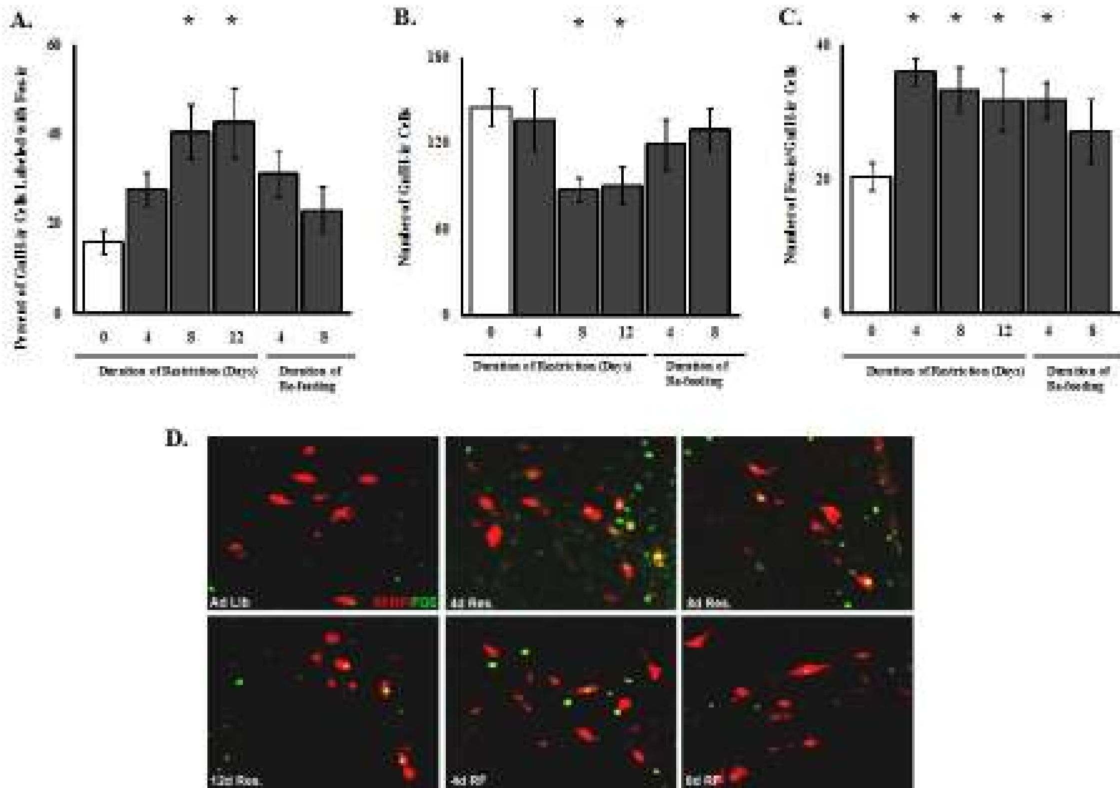
Mean and standard error of the mean of the amount of food hoarded (Top) and the number of vaginal marks produced (Bottom) in 15 min on day 3 of the estrous cycle. Each group of food-restricted female Syrian hamsters were fed 75% of their ad libitum intake for different durations before testing. Re-fed hamsters were food-restricted for 12 days and then re-fed ad libitum for 4 or 8 days. * = significantly different from hamsters fed ad libitum at $P < 0.05$.

Figure 4.2



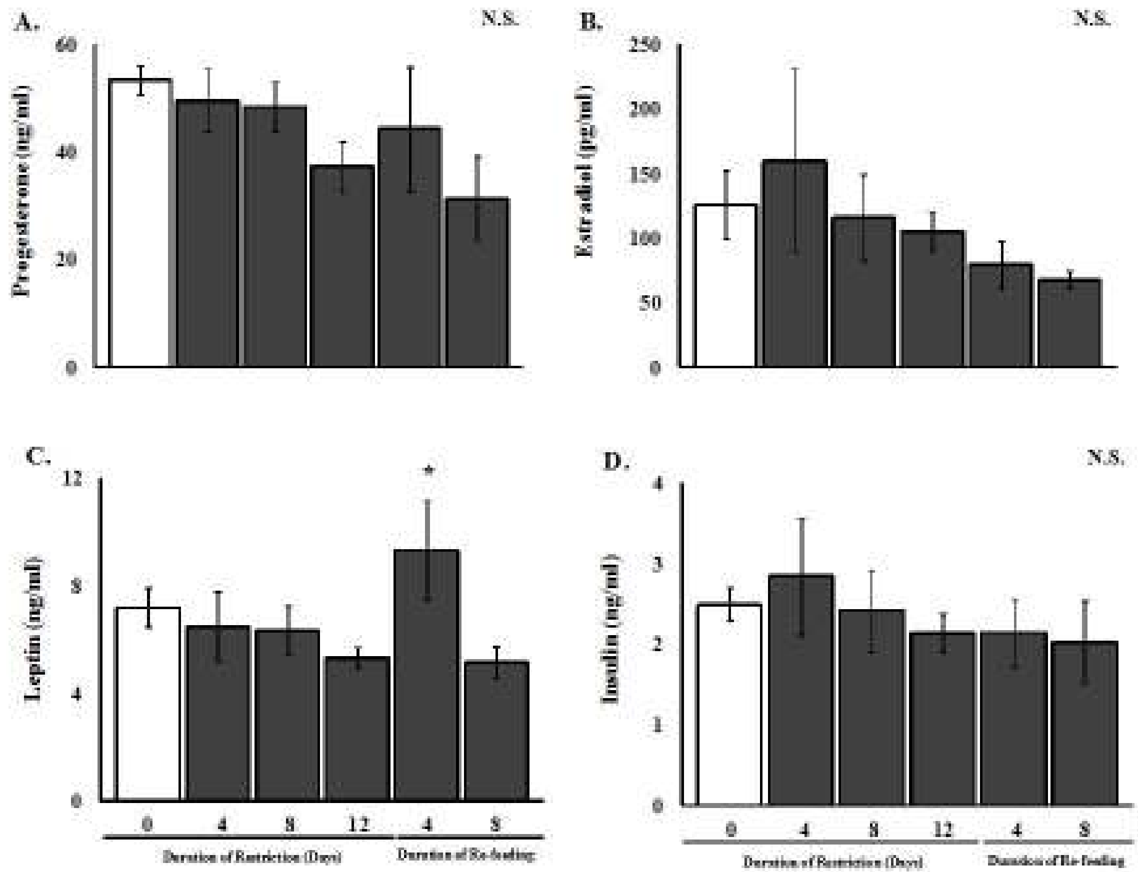
Mean and standard error of the mean for A) body weight change, B) final body weight, and C) 90-min food intake of female Syrian hamsters either fed ad libitum or food restricted to 75% of their ad libitum intake for 4, 8, or 12 days or food restricted for 12 days and then re-fed for 4 or 8 days. * = significantly different than ad libitum at P < 0.05.

Figure 4.3



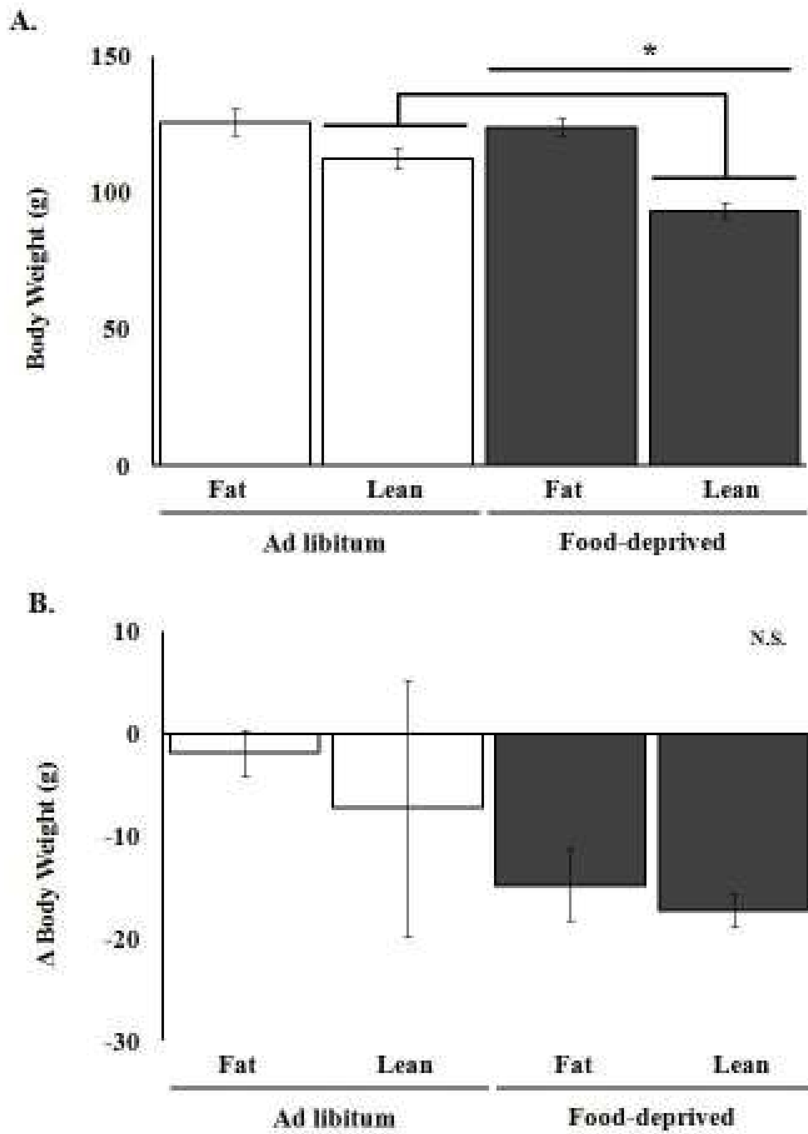
Mean and standard error of the mean for A) the percent of GnIH-Ir cells that showed Fos-Ir (the number of double-labeled Fos-Ir and GnIH-Ir cells divided by the total number of GnIH-Ir cells multiplied by 100), B) the number of GnIH-Ir cells per animal, C) the absolute number of cells that were double-labeled for both Fos-Ir and GnIH-Ir in gonadally-intact, female Syrian hamsters food restricted for different durations, and D) photomicrographs of cells double-labeled for GnIH-Ir (red) and Fos-Ir (green) following food restriction and re-feeding. Food-restricted females were fed 75% of their ad libitum intake for 4, 8 or 12 days or were food restricted for 12 days and then re-fed for 4 or 8 days. * = significantly different than ad libitum at $P < 0.05$.

Figure 4.4



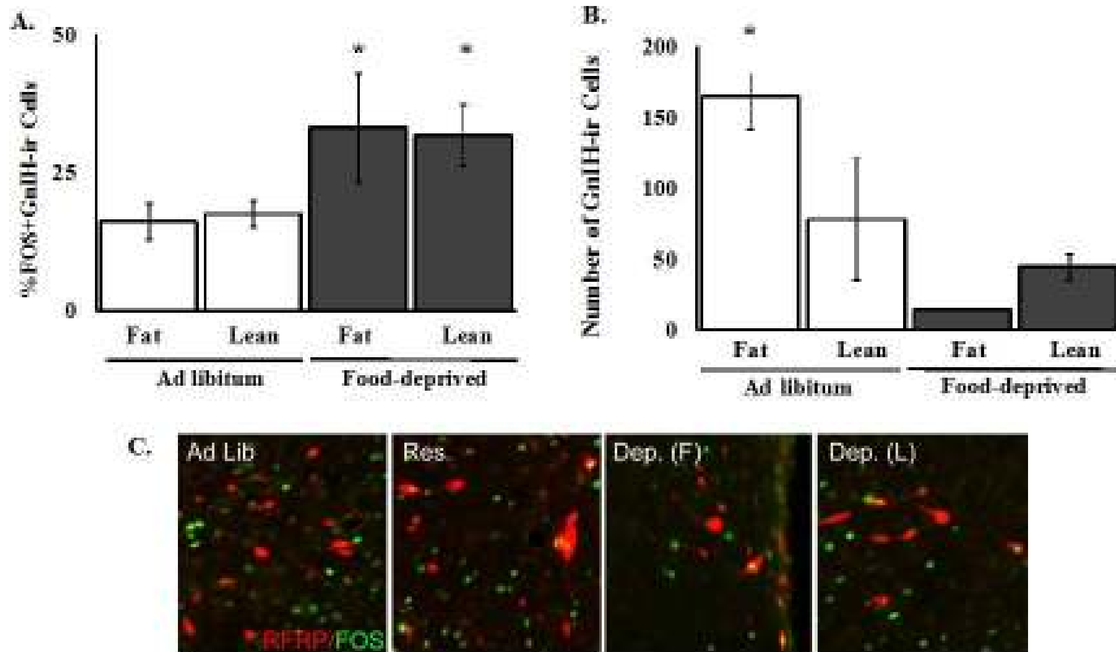
Mean and standard error of the mean for plasma concentrations of A) progesterone, B) estradiol, C) leptin, and D) insulin in female Syrian hamsters either fed ad libtum or food-restricted to 75% of their ad libitum intake at 4, 8, or 12 days after the start of food restriction or after 12 days of restriction and either 4 or 8 days of re-feeding. * = significantly different than ad libitum at $P < 0.05$.

Figure 4.5



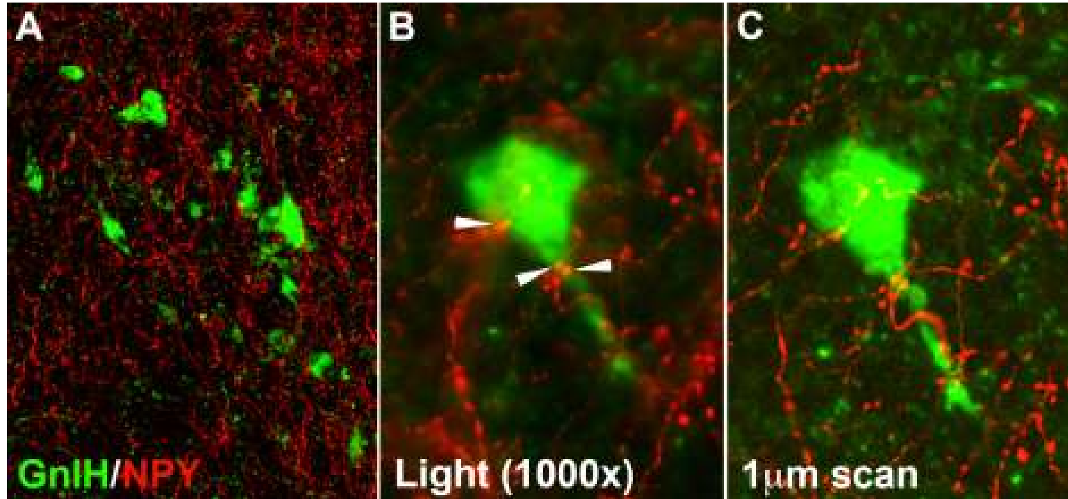
Mean and standard error of the mean for A) body weight of ad libitum-fed or food-deprived female hamsters that were lean or fat prior to the start of food deprivation and B) change in body weight of the same females after food deprivation. * = significantly different than ad libitum at $P < 0.05$.

Figure 4.6



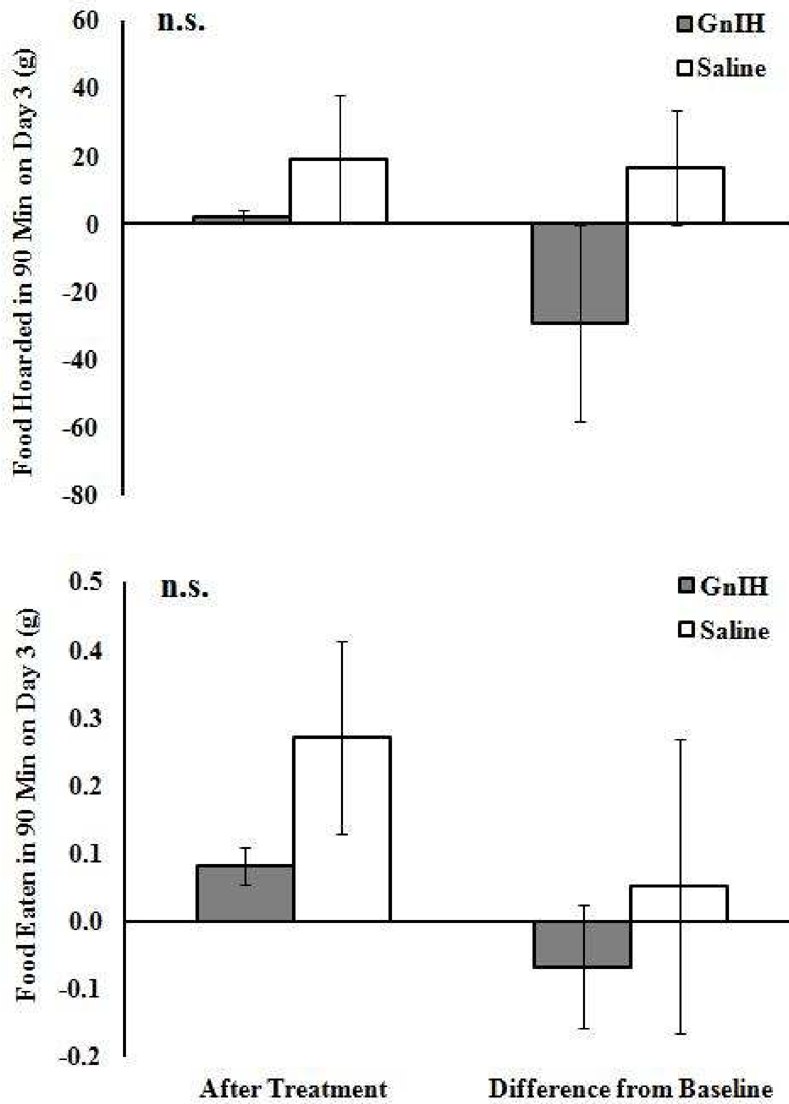
Mean and standard error of the mean for A) the number of GnIH-Ir cells in the DMH and B) the percent of GnIH-Ir cells that showed Fos-Ir in the DMH of female Syrian hamsters that were food-deprived or fed ad libitum, and half of the food-deprived hamsters were lean and the other half were fattened prior to the start of food deprivation in Experiment 4.2A. C) Photomicrographs of GnIH/Fos-Ir in the groups described. * = significantly different than ad libitum at $P < 0.05$.

Figure 4.7



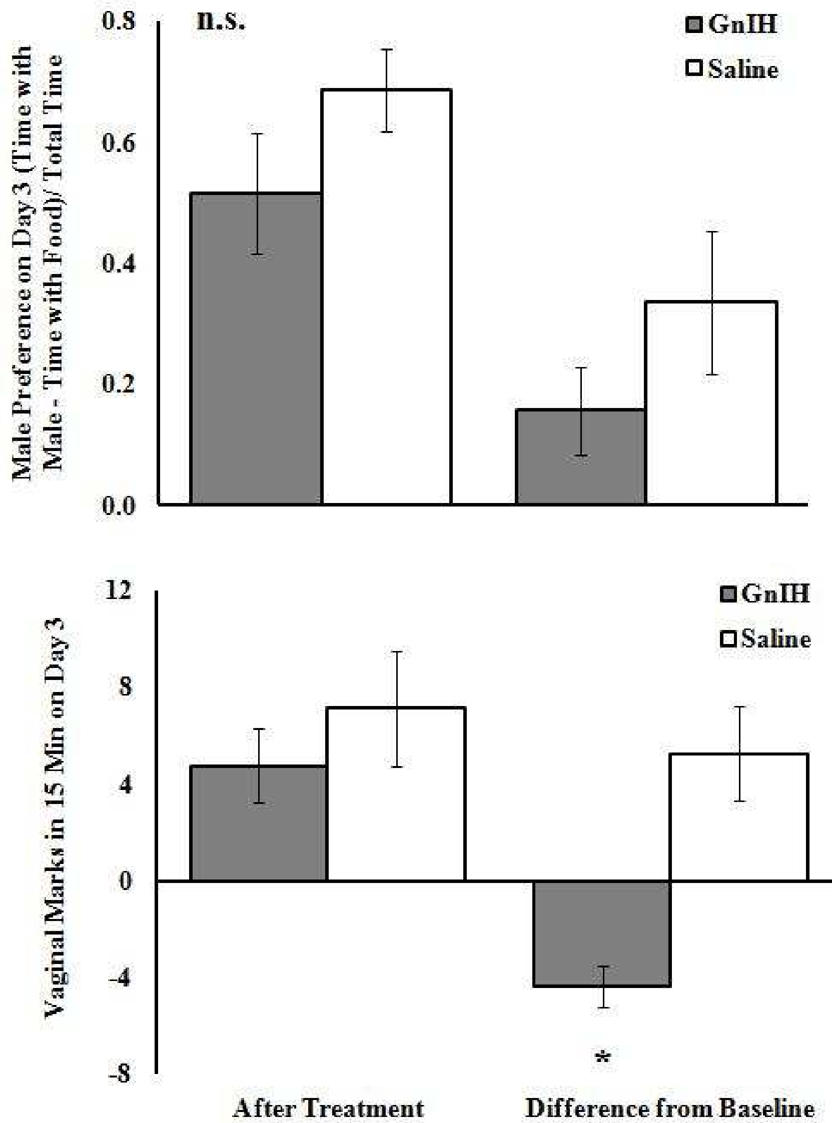
Representative photomicrographs of cells double-labeled for GnIH (red) and NPY (green). A cluster of GnIH-r cells receive extensive NPY projections at 250x in this confocal scan (A) and a single GnIH-Ir cell with presumptive NPY boutons at 1000x at the conventional (B) and confocal (C) microscopic levels.

Figure 4.8



Mean and standard error of the mean for food hoarding (Top) and food eaten (Bottom) after treatment with GnIH or saline. All females were fed ad libitum and injected with 600 ng of GnIH or saline intraperitoneally 30 min before the 15 min test in the preference apparatus. n.s. = The amount of food hoarded or eaten did not differ significantly between GnIH- or saline-treated hamsters.

Figure 4.9



Mean and standard error of the mean for male preference (Top) and vaginal marks (Bottom) after treatment with GnIH or saline. All females were fed ad libitum and injected with 600 ng of GnIH or saline intraperitoneally 30 min before the 15 min test in the preference apparatus. * Significant difference in vaginal marks subtracted from baseline at $P < 0.05$.

Chapter 5

Summary of Findings and Conclusion

The results described in this dissertation consistently support the idea that sexual and ingestive motivation are far more sensitive to low energy availability than performance or the rewarding consequences of sex. The results point to gonadotropin-inhibiting hormone (GnIH) as a candidate neuropeptide system that may be influencing behavioral motivation to switch from sexual to ingestive behavior during energetic challenges. The results are purely correlational, but it was clear that as the duration of food restriction continues, the cellular activation in GnIH cells was more closely tied to appetitive sex and ingestive behavior than it was to food intake or gonadal hormone secretion. However, it is not known whether GnIH is solely affecting food hoarding, vaginal scent marking, and male preference, or whether the peptide is acting in concert with other chemical messenger systems to elicit these effects.

The results in Chapter 2 of this dissertation confirm that sexual and ingestive motivation are more sensitive to low energy than performance. Animals fed ad libitum maintained high male preference and low food hoarding across all days of the estrous cycle. However, hamsters that were food-restricted (fed 75% of ad libitum) for 6 days prior to testing, exhibited fluctuating food hoarding and male preference across the estrous cycle with no effect on food intake. This fluctuation in behavior corresponded to

endogenous levels of estradiol, with the hormone making sex a priority around the time of ovulation.

I then directly tested whether estrous-inducing estradiol + progesterone or estradiol alone or progesterone alone affected sexual and ingestive motivation. I found that estradiol + progesterone was capable of attenuating food-restriction induced food hoarding and male preference after 4, but not 8 days of food restriction. I then tested estradiol alone and progesterone alone. In addition, I observed appetitive behaviors in the presence and in the absence of males. When males were present, females treated with estradiol alone or estradiol + progesterone had low food hoarding after 6 days of food restriction compared to females treated with progesterone. In the absence of males, however, food hoarding increased. However, after 10 days of food restriction, all groups had elevated food hoarding, even those tested in the presence of males. Despite treatment with ovarian steroids, food-restricted females showed significant changes in appetitive behaviors. Thus, it appears that food restriction affects the sensitivity to steroids rather than circulating levels of steroids. This might be due to a down-regulation of estrogen receptors in nuclei where estradiol enhances sexual behavior or an up-regulation of estrogen receptors in nuclei where estradiol enhances inhibits ingestive behavior or both. Future experiments should determine levels of restriction that affect sex but not ingestive behavior and examine estrogen receptors in the paraventricular nucleus of the hypothalamus (PVN) and perifornical area (both implicated in ingestive behavior) and ventromedial nucleus of the hypothalamus (VMH) (sex behavior).

In Chapter 3, I extended the results presented in Chapter 2 by providing a time-course of 25% food restriction to pinpoint exactly when sexual and ingestive motivation were being affected (Experiment 3.1). I found that in intact, female hamsters, food hoarding and male preference were affected between 7 and 11 days of food restriction compared to hamsters fed ad libitum. However, as in Chapter 2, food intake as well as lordosis duration were not affected until much later in restriction (20 days or more).

This dissociation between sexual motivation (vaginal marking, male preference) and performance (lordosis) led me to hypothesize that perhaps females were less motivated for sex because they were finding sex less rewarding. Furthermore, I predicted that performance would be dissociated from reward based on work from the Meisel laboratory (Meisel et al., 1996) showing that antagonists to dopamine receptors (D2) can block formation of a conditioned place preference for copulation (reward) without affecting lordosis duration. Thus, it is possible that food restriction, like dopamine antagonists, is affecting copulatory reward, but not lordosis. I tested this hypothesis using a conditioned place preference paradigm as well as by measuring neural activation within reward circuitry. I predicted that if food-restricted females found sex less rewarding, they would have failed to form a place preference or taken longer to form a place preference to copulation compared to females fed ad libitum and would have had lower neural activation in the nucleus accumbens and medial amygdala. However, in Experiment 3.3, I found that both food-restricted and ad libitum-fed females formed a place preference to copulation (spent more time on the side of the apparatus in which they received copulation) after 4 copulatory sessions, suggesting that both groups found

copulation to be positively-reinforcing. Also, in Experiment 3.2 I found that neural activation in the nucleus accumbens, as well as the medial amygdala and VMH, did not differ between mated, food-restricted and mated, ad libitum-fed females. Together, these data suggest that 25% food restriction does not affect the rewarding consequences of copulation in female Syrian hamsters. One possibility, however, is that food restriction is affecting the rewarding value of food, rather than the reward value of sex. Thus, an ideal follow-up experiment to this research would be to assess the rewarding value of food hoarding. Syrian hamsters increase food hoarding, but not food intake, following a period of reduced energy availability, so if the meso-limbic dopamine system and reward are acting to set behavioral priorities, it would be expected that food-restricted females would find food hoarding to be more rewarding compared to ad libitum-fed hamsters that fail to hoard food.

Since Chapters 2 and 3 showed that sexual and ingestive motivation are affected by 25% food restriction prior to performance or reward, I examined circulating hormones to determine whether there would be changes that corresponded with changes in behavior. In Chapter 4, I measured circulating concentrations of insulin and leptin, two hormones secreted after meals that are known to decrease food intake when administered intracerebroventricularly. These hormones are candidate chemical messengers for setting behavioral priorities because both decrease rapidly (within 12 hours) after food deprivation (Schneider et al., 2000) and affect sex and ingestive behavior (Rowland, 1978; Wade et al., 1991; Buckley and Schneider, 2003; Schneider et al., 2007).

In Experiment 4.1, I measured the levels of circulating insulin and leptin after various durations of 25% food restriction and compared this data to sex and ingestive behavior collected the previous day. For those hormones that control appetitive behavior, we would expect to see differences in the circulating levels of these hormones prior to an effect on behavior. Conversely, if changes in hormones occurred after changes in behavior, their involvement would be less likely. Similar to Chapters 2 and 3, I found that sexual and ingestive motivation were affected by 8 days of food restriction, prior to changes in food intake or circulating estradiol and progesterone. However, contrary to our prediction, 25% food restriction alone did not result in significant decreases in plasma insulin or leptin concentrations. Thus, changes in vaginal scent marking, male preference and food hoarding were not explained by changes in these hormones.

Because circulating leptin and insulin could not account for changes in behavior in Experiment 4.1, I tested another chemical messenger capable of influencing ingestive and sex behavior, GnIH. GnIH cells are neuroanatomically-positioned in such a way to regulate sex and ingestive behavior. For instance, GnIH cells from the dorsomedial nucleus of the hypothalamus (DMH) have been shown to project to putative anorectic and orexigenic-containing cells in the PVN and Arc including NPY, proopiomelanocortin, orexin, and melanin-concentrating cells in the sheep and gonadotropin-releasing hormone (GnRH) neurons in the hamster (Clarke et al., 2008; Kriegsfeld et al., 2006). Central or peripheral injections of GnIH rapidly suppress LH and GnRH secretion in hamsters, mice, rats, and sheep, decrease copulatory solicitation displays by female sparrows, and suppress sex behavior of male rats (mounting,

intromissions, and ejaculations) (Anderson et al., 2009; Clarke et al., 2008; Johnson et al., 2007; Kriegsfeld, 2006; Murakami et al., 2008; Tsutsui et al., 2010). Like other peptides that decrease aspects of reproduction, injections of GnIH have been shown to increase food intake in male rats, sheep, and monkeys (I.J. Clarke personal communication; Johnson et al., 2007; Murakami et al., 2008).

Since changes in circulating leptin, insulin, ovarian steroids, and the meso-limbic dopamine system could not account for changes in sexual and ingestive motivation, I hypothesized that perhaps GnIH was influencing these behaviors. Like the aforementioned messenger systems, if GnIH was acting to set behavioral priorities, we predicted that we would see a change in the population of cells in the DMH prior to a change in behavior. In this experiment (Experiment 4.1), I discovered a positive association between cellular activation of GnIH cells and food hoarding, but not food intake, in the female Syrian hamster. While rats increase food intake in response to GnIH, they are also a species that, unlike Syrian hamsters, exhibits post-fast hyperphasia (Kutscher, 1969). Instead of overeating in response to energetic challenges, Syrian hamsters actually over-hoard with a few exceptions including during the energetic demands of lactation or after treatment superphysiological insulin (Buckley and Schneider, 2003; Fleming and Miceli, 1983; Phillips et al., 1989; Wade et al., 1991; Wong, 1984b). Thus, the pattern of GnIH expression and food hoarding that both increased the longer hamsters were food-restricted and decreased following re-feeding, is in agreement with the ingestive behavior of this species.

While cellular activation of GnIH was positively associated with food hoarding in Experiment 4.1, the peptide was negatively associated with vaginal scent marking, but not circulating estradiol or progesterone. Although vaginal marking can be induced with estradiol, vaginal marking is an appetitive sexual behavior that is more sensitive to low energy than circulating ovarian steroids or lordosis in the Syrian hamster (Takahashi et al., 1985; Schneider et al., 2007). Thus, it was not surprising that GnIH was correlated with this appetitive sex behavior and not estradiol and progesterone. Although the hamsters in this experiment were sacrificed before they were tested for sexual receptivity, it is likely that all of the food-restricted females would have shown lordosis because levels of estradiol and progesterone did not differ between food-restricted and ad libitum-fed hamsters after 12 days of restriction, and the display of the lordosis reflex is dependent on physiological estradiol and progesterone in this species (Lisk and Reuter, 1980).

While cellular activation in GnIH cells increased with 25% food restriction and decreased following re-feeding, the mean number of GnIH cells displayed the opposite pattern, decreasing with restriction and increasing with re-feeding. A reduction in GnIH cell number is consistent with the idea that energy deficits cause increases in GnIH secretion without compensatory increases in GnIH synthesis, especially since the number of GnIH cells returned to baseline after re-feeding. Thus, it is unlikely that this population of cells is dying, but rather not being detected by GnIH-ir because the protein had already been secreted at the synapse. A similar phenomenon has been observed in other populations of hypothalamic neurons. For instance, castration, like food restriction,

decreases immunoreactivity in discrete populations of neurons (NPY, cholecystokinin, substance-P) in the hypothalamus and like re-feeding, is restored by testosterone treatment (J.M. Swann personal communication; Sahu et al., 1989; Simerly and Swanson, 1987; Swann and Newman, 1992).

While I found a significant association between GnIH and food hoarding and vaginal marking in 25% food-restricted females in Experiment 4.1, peripheral injections of GnIH to ad libitum-fed hamsters in Experiment 4.3 did not cause the expected changes in sex and ingestive behavior. However, exogenous GnIH also did not suppress the LH surge and thus, it is likely that the GnIH that were given was inactive, perhaps due to shipping from Japan. Other experiments showed that the dose of GnIH we used significantly inhibited LH secretion. Future experiments should continue to examine the role of GnIH in setting behavioral priorities in areas where energy availability fluctuates, using agonists and antagonists. Prior to the start of an experiment similar to this, GnIH should be tested for its efficacy in suppressing the LH surge (Kriegsfeld et al., 2006). GnIH and an antagonist to its receptor (RF9), like estradiol + progesterone in Chapter 2, should then be tested on ingestive and sex behavior in the presence and absence of potential mating partners to assess if and how this peptide may be playing a role in setting behavioral priorities. I would expect that like estradiol + progesterone, RF9 would decrease food hoarding in the presence of males, but would have little suppressive effect on food hoarding when the male was removed.

Lastly, in Experiment 4.2, I found that NPY cell terminals were found in close apposition to GnIH cells. NPY is a potent orexigenic peptide produced and secreted

mainly from the hypothalamus and nucleus of the tractus solitarus in rodents (Chronall et al., 1985; de Quidt et al., 1986) that increases in response to energy restriction and stimulates ingestive behavior in many species including goldfish, snakes, chicks, mice, rats, rabbits, sheep, pigs, Siberian hamsters, and the subject of the current experiments, Syrian hamsters (Bartness, 2003; Boss-Williams and Bartness, 1996; Brady et al., 1990; Clark et al., 1984; Day et al., 2005; Jones et al., 2004; Kuenzel et al., 1987; Kulkosky et al., 1988; Lopez-Patino et al., 1999; Miner et al., 1989; Morley et al., 1987a; Morley et al., 1987b; Morris and Crews, 1990; Parrott et al., 1986; Pau et al., 1988; Reddy et al., 1999; Sahu et al., 1988a; Schwartz et al., 1998; Stanley and Leibowitz, 1984; White and Kershaw, 1990). NPY also has opposing effects on reproduction and suppresses LH secretion and causes anestrus when injected into rats, hamsters, rabbits, and monkeys (Clark et al., 1985; Corp et al., 2001; Jones et al., 2004; Kaynard et al., 1990; Khorram et al., 1987; McDonald et al., 1989; Raposinho et al., 1999). Thus, NPY represents an ideal candidate peptide that is responsive to low energy and may provide a signal of energy status to GnIH cells within the DMH. Future experiments should assess, perhaps, how other chemical messenger systems, such as estradiol, interact with NPY to influence sexual and ingestive motivation in the female, Syrian hamster.

In conclusion, the data presented in this dissertation provide novel evidence that mild food restriction effects sexual and ingestive motivation prior to performance or copulatory reward. Evidence supports the idea that food restriction makes female hamsters less sensitive to the anorectic effects of estradiol, and is likely mediated by a peptide system that includes GnIH. Cellular activation of GnIH cells increased in

response to food restriction and was associated with increases in food hoarding and decreases in sexual motivation. One possibility is that under normal energy conditions or around the time of the LH surge, estradiol inhibits GnIH cells, thereby suppressing ingestive behavior and stimulating reproductive activities. However, reduced energy availably, possibly detected by NPY neurons that project to GnIH cells, triggers a down-regulation of estradiol receptors on GnIH cells, releasing the cells from inhibition, and causing the release of GnIH. GnIH then increases the motivation for food and decreases the motivation for sex. Collectively, it is likely that GnIH is part of a system that is important for setting behavioral priorities in environments where energy availability fluctuates.

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Delaware Valley College — Doylestown, PA

Introduction to Laboratory Animal Science — 2003

Employment and Work Experience

Children's Hospital of Philadelphia — Philadelphia, PA

Cardiology Department — 2004 to 2005

Research Technician, Level II

Delaware Valley College — Doylestown, PA

Student Animal Laboratory Manager — 2002 to 2004

Delaware Valley College — Doylestown, PA

Animal Laboratory Technician — 2001

Grants and Awards

Gordon C. Thorne Fellowship, Lehigh University	2011
Lehigh University travel award	2011
Graduate student spotlight, Lehigh University	2011
Grants-in-aid of research, Sigma Xi	2010
Cover photograph for Hormones and Behavior, Vol. 58, Issue 4	2010
Gordon C. Thorne Fellowship, Lehigh University	2010
Lehigh University travel award	2010
Craig Hill achievement award, Delaware Valley College	2004
Delta Tau Alpha honors society, Delaware Valley College	2003 to 2004
Dean's list, Delaware Valley College	2001 to 2004
Faculty scholarship, Delaware Valley College	2000 to 2004

Publications

Klingerman, C.M.❖, W.P. Williams❖, L.J. Kriegsfeld, and J.E. Schneider. (Accepted with revisions) "Food restriction-induced changes in gonadotropin-inhibiting hormone cells are associated with changes in sexual motivation and food hoarding, but not sexual performance and food intake." *Frontiers in Systems and Translational Endocrinol.* ❖Co-first authors on this manuscript.

Schneider, J.E., **C.M. Klingerman**, and A.A. Abdulhay. (Accepted with revisions) "Sense and nonsense in metabolic control of reproduction." *Frontiers in Systems and Translational Endocrinol.*

Klingerman, C.M., A. Patel, V.L. Hedges, R.L. Meisel, and J.E. Schneider. (2011) "Food restriction dissociates sexual motivation, sexual performance, and the rewarding consequences of copulation in female Syrian hamsters." *Behav Brain Res.* 223:356-370.

Klingerman, C.M., Krishnamoorthy, K., Patel, K., Struby, C. Spiro, A.B., and Schneider, J.E. (2010) "Energetic challenges unmask the role of ovarian hormones in orchestrating ingestive and sex behaviors." *Horm. Behav.* 58:563-574.

Kung, Jr., L., E. Stough, E. McDonell, R.J. Schmidt, M. Hofherr, L., Reich, and **C. Klingerman**. (2010) "The effect of wide swatching on wilting times and nutritive value of alfalfa haylage." *J. Dairy Sci.* 93:1770-1773.

Klingerman, C.M., W. Hu, E.E. McDonell, M.C. DerBedrosian, and L. Kung, Jr. (2009) "An evaluation of exogenous enzymes with amylolytic activity for dairy cows." *J. Dairy Sci.* 92:1050-1059.

Hu, W., R.J. Schmidt, E.E. McDonell, **C.M. Klingerman**, and L. Kung, Jr. (2009) "The effect of *Lactobacillus buchneri* 40788 or *Lactobacillus planterum* MTD-1on the

fermentation and aerobic stability of corn silages ensiled at two dry matter contents." *J. Dairy Sci* . 92:3907-3914.

<u>Presentations at National and International Meetings</u>

Klingerman, C.M., Patel, A., Hedges, V.L., Meisel, R.L., and Schneider, J.E. "Energetic deficits dissociate motivation from performance and reward." (talk) *Society for the Study of Ingestive Behavior*. Clearwater, FL. 2011.

Williams, W.P., **C.M. Klingerman**, J. Simberlund, N. Brahme, L.J. Kriegsfeld, and J.E. Schneider. "Energetic and reproductive status impact RFamide-related peptide-3 immunoreactivity in female Syrian hamsters." (poster) *Society for Neuroscience.* San Diego, CA. 2010.

Klingerman, C.M., Patel, A., Hedges, V.L., Meisel, R.L., and Schneider, J.E. "Food restriction alters appetitive and ingestive behaviors but not consummatory behaviors nor neural activation in the ventromedial nucleus of the hypothalamus and nucleus accumbens." (poster) *Society for Behavioral Neuroendocrinology.* Toronto, Canada. 2010.

Schneider, J.E., **C.M. Klingerman**, K. Krishnamoorthy, K. Patel, C. Struby, and A.B. Spiro. "Energetic challenges unmask the role of ovarian hormones in orchestrating the

appetitive ingestive and sex behaviors, food hoarding, and paracopulatory behaviors." (poster) *Society for Behavioral Neuroendocrinology.* Toronto, Canada. 2010.

Patel, A., **C.M. Klingerman**, R. Meisel, J.E. Schneider. "Dopamine and the desire for food and sex." (poster) *Society for Behavioral Neuroendocrinology.* East Lansing, MI. 2009.

Klingerman, C.M., W. Hu, E.E. McDonell, M.C. DerBedrosian, and L. Kung, Jr. "An evaluation of exogenous enzymes with amylolytic activity for dairy cows." (poster) *American Dairy Science Association and American Society of Animal Science joint annual meeting.* Indianapolis, IN. 2008.

Klingerman, C.M., J. Simberland, R. Shankar, C. Casper, and J. Schneider. "Detailed analysis of effects of energy on ingestive and sex behaviors." (poster) *Society for Behavioral Neuroendocrinology.* Groningen, The Netherlands. 2008.

Klingerman, C.M., R.J. Schmidt, W. Hu, E.E. McDonell, and L. Kung, Jr. "The effect of microbial inoculants on the fermentation and aerobic stability of orchard grass silage." (talk) *American Dairy Science Association and American Society of Animal Science joint annual meeting.* San Antonio, TX. 2007.

Klingerman, C.M., R.J. Schmidt, W. Hu, E.E. McDonell, and L. Kung, Jr. "The effect of microbial inoculants on the fermentation and aerobic stability of orchard grass silage." (poster) *American Dairy Science Association and American Society of Animal Science joint annual meeting.* San Antonio, TX. 2007.

Kung, L. Jr., E.C. Stough, E.E. McDonell, R.J. Schmidt, M.W. Hoffher, L.J. Reich, and **C.M. Klingerman**. "The effect of wide swathing on wilting times and nutritive value of alfalfa haylage." (poster) *American Dairy Science Association and American Society of Animal Science joint annual meeting.* San Antonio, TX. 2007.

McDonell, E.E., **C.M. Klingerman**, R.J. Schmidt, W. Hu, and L. Kung, Jr. "An evaluation of two methods to cover bunker silos to maintain the nutritive value of silage." (talk) *American Dairy Science Association and American Society of Animal Science joint annual meeting.* San Antonio, TX. 2007.

Schmidt, R.J., J.A. Mills, W. Hu, **C.M. Klingerman**, E.E. McDonell, and L. Kung, Jr. "Changes in fermentation end products and use of real-time quantitative PCR to monitor the dynamics of *Lactobacillus buchneri* in alfalfa silage." (poster) *American Dairy Science Association and American Society of Animal Science joint annual meeting.* San Antonio, TX. 2007.

Schmidt, R.J., W. Hu, **C.M. Klingerman**, E.E. McDonell, and L. Kung, Jr. "The effect of *Lactobacillus buchneri* 40788 with or without *Pediococcus pentasaceous* on the fermentation and aerobic stability of corn silage made at different locations." (talk) *American Dairy Science Association and American Society of Animal Science joint annual meeting.* San Antonio, TX. 2007.

Yellen, B.B., M. Chorney, I. Fishbein, N. Dai, **C.M. Klingerman**, I. Alferiev, O. Nyanguile, R. Wilensky, G. Friedman, and R.J. Levy. "Site specific gene delivery using magnetic forces to localize adenoviral vector-magnetic nanoparticle complexes to stented arterial segments." (poster) *American Heart Association Scientific Sessions.* Dallas, TX. 2005.

Yellen, B.B., M. Chorney, I. Fishbein, D.N. Williams, **C.M. Klingerman**, I.S. Alveriev, O. Nyanguile, G. Friedman, and R.J. Levy. "Nanoparticle mediated gene delivery to magnetized implants." (poster) *American Society for Gene Therapy.* St. Louis, MO. 2005.