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The Microstructure of Food Intake under conditions of High-Fat Diet, Social Stress and Social Subordination

A dissertation submitted to the

Division of Research and Advanced Studies
of the University of Cincinnati

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY (Ph.D.)

in the Graduate Program in Neuroscience
of the College of Medicine

2009

By

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Abstract

The pattern of food consumption can influence body weight and composition. This dissertation utilizes a recently developed meal pattern program created for the studies presented here to examine how high-fat diet and social stress alter the pattern of food consumption and how these changes, along with individual-vulnerability to stress, may contribute to known diet and stress-related effects on body weight and composition.

The established meal pattern program was validated by exposing rats to a high-fat diet, which resulted in the gain of body weight and adipose tissue and a decreased meal frequency and increased meal size. Furthermore, meal size was shown to have a positive correlation with the gain of adipose mass. All of these results have been previously reported allowing us to verify the system for use in our laboratory model of chronic social stress: the visible burrow system (VBS) and for the first time measure meal patterns during social stress exposure. Dominant (DOM) animals lose a minimal amount of weight and adipose tissue during VBS exposure, but quickly recover their weight and body composition to controls (CON) levels once removed from the VBS. Subordinate (SUB) animals lose a significant amount of body weight, adipose and lean tissue during VBS housing, and upon recovery from stress regain body weight preferentially as adipose tissue.
DOM and SUB are hypophagic during initial exposure to the VBS. DOM initially had a reduced meal frequency, but once the hierarchy was established showed no signs of disrupted ingestive behavior. SUB took smaller, fewer meals during the formation of the hierarchy, but once stable, only took fewer meals although had other signs of disrupted ingestive behavior such as increased meal duration, Intrameal interval and Intermeal interval along with an interrupted circadian pattern of feeding suggested by their increased meal frequency in the light cycle. These changes are likely a result of adaptation to the VBS environment. Expression of neuropeptide Y (NPY), a potent orexigenic agent expressed in the hypothalamus, was increased immediately following VBS exposure in both the DOM and SUB population suggesting that another mechanism is blocking or impairing the NPY signal to stimulate food intake during VBS housing.

During recovery, DOM and SUB were hyperphagic. SUB accomplished this through taking larger meals. This in combination with the slight decrease in meal number likely contributes to the gain in adipose tissue present in this population following VBS exposure. Furthermore, the circadian pattern of feeding remained altered during the initial recovery period and meal duration and intermeal interval were longer than DOM and CON throughout the 3-week recovery period. After 1- and 3-weeks of recovery hypothalamic NPY expression was not different among the groups suggesting that, in this case, NPY does not mediate the hyperphagia following VBS exposure.
Together, these studies suggest that pattern of ingestive behavior is an important factor in the body weight and composition changes associated with the VBS models of chronic social stress; however, individual vulnerability may also contribute to these changes. The OMEGA phenotype, as established through behavior within the VBS, loses a considerable amount of body weight, adipose and lean tissue and is hyporesponsive to a novel acute restraint stress test. An increase in NPY expression in the central and basolateral nucleus suggest an impaired anxiety mechanism in this maladaptive phenotype.
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<tbody>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AgRP</td>
<td>agouti-related peptide</td>
</tr>
<tr>
<td>Arc</td>
<td>arcuate nucleus of the hypothalamus</td>
</tr>
<tr>
<td>BLA</td>
<td>basolateral nucleus of the amygdala</td>
</tr>
<tr>
<td>CCK</td>
<td>cholecystokinin</td>
</tr>
<tr>
<td>CeA</td>
<td>central nucleus of the amygdala</td>
</tr>
<tr>
<td>CGL</td>
<td>corrected gray level</td>
</tr>
<tr>
<td>CON</td>
<td>control</td>
</tr>
<tr>
<td>CORT</td>
<td>corticosterone</td>
</tr>
<tr>
<td>CR</td>
<td>consumption rate</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotropin-releasing hormone</td>
</tr>
<tr>
<td>DIO</td>
<td>diet-induced obesity</td>
</tr>
<tr>
<td>DMH</td>
<td>dorsomedial nucleus of the hypothalamus</td>
</tr>
<tr>
<td>DOM</td>
<td>dominant</td>
</tr>
<tr>
<td>EPM</td>
<td>elevated plus maze</td>
</tr>
<tr>
<td>FST</td>
<td>forced swim test</td>
</tr>
<tr>
<td>GC</td>
<td>glucocorticoid</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalamic-pituitary adrenal (axis)</td>
</tr>
<tr>
<td>Intra-MI</td>
<td>intrameal interval</td>
</tr>
<tr>
<td>Inter-MI</td>
<td>intermeal interval</td>
</tr>
<tr>
<td>MeA</td>
<td>medial nucleus of the amygdala</td>
</tr>
<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus of the hypothalamus</td>
</tr>
<tr>
<td>REM</td>
<td>rapid-eye movement</td>
</tr>
<tr>
<td>SFC</td>
<td>surface chamber (of VBS)</td>
</tr>
<tr>
<td>SUB</td>
<td>subordinate</td>
</tr>
<tr>
<td>VBS</td>
<td>visible burrow system</td>
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Chapter 1

General introduction and background.
The development of the Westernized world has brought about advancement in technology, agriculture, medicine, business, politics, economics, industry and much more. With these advancements came the progression of disease and health problems which can be attributed, at least in part, to these westernized changes. Our diet has become more palatable, calorically-dense and easily attainable; lifestyles are more sedentary and the combination of these is often attributed to the development of obesity. Another aspect of our advanced lifestyle is the incorporation of stress into our daily lives. Whether from our job, our family or another source, the level of daily psychological stress is on the rise. Long term, or chronic, exposure to stress can lead to many of the same complications associated with obesity, including obesity itself.

As our society, and world, continues to progress, it is important to understand the relationships among our lifestyle and related-diseases. Our environment of readily available, palatable, calorically-dense foods often leads to overconsumption, weight gain and adiposity. Similarly, exposure to psychological stress has implications on food intake, body weight and body composition. It is well known that rapid fluctuations in weight or the gain of weight, particularly adipose tissue, has detrimental effects on the endocrine and metabolic health of an individual. At the root of these changes is the pattern of food consumption, as this basic behavior has great impact on body weight and composition. This dissertation explores the role of high-fat diet and exposure to social stress on meal patterns to understand how these experiences may alter meal patterns, which in turn influence body weight and composition.
The fundamental unit of ingestive behavior: the meal

The idea of a meal seems straightforward at face value. Meals are consumed on a regular basis in our society generally as breakfast, lunch and dinner each day. Meals are an important behavior in all cultures and ethnic backgrounds and are consumed by people of all ages. These generalities transcend to the animal kingdom, as all animals need nutrients to survive, and nutrients are provided by intake of individual meals.

The precise measurement of meals has been considered important since the study of spontaneous behavior began. The seminal work of Curt Richter provides invaluable insight into the natural behavior of many species, and through his creative approach to experimental design we now benefit from his early conclusions.

Richter began in his graduate years by studying the spontaneous activity of his experimental animal, the domestic laboratory rat, by placing a smoked drum beneath an individual rat’s cage that would indicate the animal’s movements in real time by marks on the smoked surface. This led to the discovery that the natural activity of an animal occurs rhythmically and that this rhythmic pattern had to be, at least in part, controlled by internal factors as the 24-hour environment was kept in complete light and as constant as possible. Furthermore, Richter observed that the rat’s activity level changed based on the presence of food. This led to the design of the ‘double cage’ and the first measurements of the patterns of food intake. Similar to the activity-monitoring cage placed above a smoked drum, the double cage was a series of two independent
cages set upon separate tambours that were connected by a narrow tunnel through which only a rat's head could fit. This allowed each cage to be monitored separately in order to independently evaluate activity and ingestive behavior in the same rat during the same period of time, and it also allowed for the determination of the relationship between these two behaviors. Based on such data, Richter concluded that activity and ingestion are closely related. Follow-up studies to examine the ‘food habits’ of the rat led to the observation that rats consume food in specific bouts and that individual rats are consistent in their pattern from day to day (Richter 1927; Blass 1976; Moran and Tamashiro 2007).

Based on these, and many other ingenious studies, Richter concluded that the spontaneous behaviors of animals contribute to the maintenance of homeostasis (Moran and Tamashiro 2007; Woods and Ramsay 2007). Richter’s conclusions could then be integrated with those established by Claude Bernard and Walter Cannon, who are credited with the concept of homeostasis, whereby the body maintains a constant internal environment through physiological and behavioral mechanisms (Cannon 1929; Woods and Ramsay 2007).

Ingestive behavior is one essential component to the maintenance of a homeostatic balance as it provides nutrients to the body. These nutrients are crucial in maintaining available energy stores and supplying essential building blocks such as amino acids, saccharides and fatty acids for multiple functions throughout the body. As
described by Richter, animals ingest nutrients in bouts, or meals, leaving the meal to be
the fundamental measurable unit of ingestive behavior.

Measuring a meal

Although the meal is the basic component of food intake, studies in ingestive
behavior seldom describe meal patterns. As recently discussed by Geary (Geary
2005), this likely results from the complications associated with the study of meal
patterns. Some of these complications include the lack of a uniform definition of the
meal, lack of consistent results and sensitivity of results to many factors including
environmental influences, such as cage changing.

In order to measure meal patterns, some type of continual monitoring device
must be employed. Richter utilized the smoke-covered drum to obtain a continual
measure, but computers are now used to acquire this information. This results in a form
of raw data. These data need to be filtered through some type of meal definition as
rats, like humans, do not continually consume food without pausing perhaps for a drink
and returning to the same bout of consumption. The definition of a meal is inconsistent
throughout the literature. Most include some combination of minimum meal size,
minimum meal length and minimum intermeal interval. Selecting strict criteria is crucial
to the conclusion of any experiment as evaluating the same data with two different
definitions can yield meaningfully different results (Castonguay, Upton et al. 1982;
Castonguay, Kaiser et al. 1986). Many have attempted to apply statistical analysis,
logarithmic transformations and break points to suggest the best way to define a meal
(De Castro 1975; Slater and Lester 1982; Demaria-Pesce and Nicolaidis 1998; Zorrilla, Inoue et al. 2005), nonetheless, the literature remains variable.

The lack of consistent results is a reflection of the variability in the meal pattern definition. This relationship became apparent when some proposed a postprandial relationship between meal size and subsequent intermeal interval suggesting that the larger the meal taken, the longer the time before the next meal (Thomas and Mayer 1968; De Castro 1975; Danguir 1979). Others refuted this concept (Panksepp 1973; Castonguay, Kaiser et al. 1986; Collier, Johnson et al. 1999). Today, with the study of different macronutrients and other experimental manipulations it has been suggested that this relationship is dependent on many factors, such as palatability and the macronutrient composition.

Controls of the meal and of meal patterns

From his early work, Richter suggested that food intake, and therefore meals, are controlled by internal cues. Today we appreciate a complex neuroendocrine system, which controls and mediates ingestive behavior.

Animals at their homeostatic body weight and in a predictable, stable environment are able to adjust their meal taking strategy to have a daily caloric intake
sufficient to maintain body weight, even in the face of increasing or decreasing energy
demands. For example, a lactating rat requires more energy to sustain its body weight.
To account for this, meal size is first increased followed by an increased frequency if the
former did not successfully allow the maintenance of body weight (Strubbe and
Gorissen 1980; Woods and Strubbe 1994). In the case of an unpredictable
environment, animals also have the ability to adjust their behavior, although not always
as successfully. Collier and colleagues (2004) suggested nine priorities of ingestive
behavior for animals in a natural setting, priorities which may be competitive and/or
unpredictable. These include initiating the meal, detecting a food source, acquiring the
food supply, consuming the food, ending the meal, and digesting the food, all while
avoiding interactions with potential predators or competitors, maintaining energy
balance and economizing (Collier and Johnson 2004). These priorities cause the
animal to evaluate the cost of procurement, meaning the risk and energy demands
required to perform these necessary tasks for ingestive behavior success. Animals
successful in this endeavor are able to adjust their meal taking approach, for example
by increasing meal size and decreasing meal frequency and thereby reducing the many
risks associated with ingestive behavior (Collier and Johnson 2004).

If animals are in a stable environment, they can learn to predict or anticipate
meals by making physiological responses. The most classic example is the work of
Ivan Pavlov in dogs. He observed that dogs would salivate at the mere presence of the
lab technician who had fed them on a daily basis. Although this is the foundation of
classical conditioning, it can also represent a physiologic anticipatory response for a meal.

Although it is controversial, blood glucose levels have been reported by some to slowly decrease prior to a meal. From this Le Magnen and others proposed that a meal was the result of a sensed energy deficit which was signaled by the drop in preprandial glucose leading to the depletion-repletion theory of food intake (Le Magnen and Devos 1970; Campfield, Brandon et al. 1985; Campfield and Smith 1990). Woods and colleagues (1994) refute this theory suggesting that, in a predictable environment, animals learn to anticipate a meal and that the drop in glucose is an example of this expectation. The premise is that a rat which is maintaining homeostasis in an ad libitum-fed situation would have sufficient energy reserves to survive and would never reach a state of a metabolic emergency as energy reserves could be broken down to provide the needed glucose suggested by the depletion-repletion theory. Hence, Woods (Woods and Strubbe 1994) suggests that the drop in glucose is an anticipatory physiological response to the impending meal, one that prevents the animal from developing hyperglycemia following food consumption, thus maintaining metabolic homeostasis. Furthermore, cephalic insulin is secreted prior to a meal to aid in the prevention of hyperglycemia. Both of these responses occur when a meal is expected, even if food is not present, and do not occur again until the time in which another meal would normally be taken despite missing the previous meal (Campfield and Smith 1990). Additionally, if the cephalic insulin response is blocked, animals take smaller meals and develop glucose intolerance (Berthoud, Bereiter et al. 1981; Power and
Schulkin 2008) supporting the idea that these anticipatory changes in glucose and insulin aid in the maintenance of homeostasis by allowing larger meals to be taken to meet energy demands without the damaging effects of hyperglycemia.

Meal size can be considered the output measure of meal termination. Early studies in the sham-fed rat by Davis, in which a gastric fistula was placed in the stomach and contents were aspirated out or simply drained from the stomach during feeding, indicate that the signals which act to terminate a meal are postingestive as sham-fed rats had an excessively large meal size (Davis and Campbell 1973; Young, Gibbs et al. 1974). These results led to the theory that the control of meal size is a result of a balance between positive feedback from orosensory stimuli promoting food intake and negative feedback from postingestive stimuli inhibiting food intake and when the negative feedback became stronger the meal would cease (Davis, Collins et al. 1975; Davis and Levine 1977). Whether providing positive or negative feedback, these stimuli act throughout the digestive tract in a direct manner as they stimulate preabsorptive receptors which provide information to the hindbrain via afferent fibers. It was further suggested by Gerard Smith that not only do direct controls exist on meal size, but indirect controls also influence the termination of a feeding bout. Indirect controls include influences that do not act on preabsorptive receptors. These range from circadian fluctuations in circulating hormones to conditioned preferences to social and cultural traditions (Smith 1996; Smith 2000).
Current literature now focuses on the role of gut hormones, satiation factors, adiposity signals and neuropeptides in the (indirect) control of food intake. However, Gerard Smith was first to identify a gut peptide, cholecystokinin (CCK), which controlled food intake specifically through meal termination (Gibbs, Young et al. 1973). The authors of this influential paper also proposed criteria to determine if a particular peptide played a role in meal taking behavior, criteria which are still pertinent today. Many signals have now been identified as involved in the control of ingestive behavior including: leptin, insulin, CCK, amylin, glucagon-like peptide 1, ghrelin, glucocorticoids and others; many of which act within the hypothalamus, and other brain regions, to modulate neuropeptide expression (Schwartz, Woods et al. 2000; Woods, Schwartz et al. 2000; Woods and Seeley 2000; Valassi, Scacchi et al. 2008; Woods and D'Alessio 2008).

One of the modulated neuropeptides is neuropeptide Y (NPY). NPY is a 36-amino acid peptide originally isolated from porcine brain and is a member of the pancreatic polypeptide family (Tatemoto, Carlquist et al. 1982; Colmers and Wahlestedt 1993). NPY is ubiquitously expressed throughout the central nervous system, but has high concentrations in the hypothalamus where it exerts its most well known function of stimulating food intake (Kalra and Kalra 2004). Many of the above-mentioned hormones exert their function by acting on NPY neurons within the arcuate nucleus, eliciting an orexigenic effect with an increase in meal size and frequency (Marin Bivens, Thomas et al. 1998; Moran, Katz et al. 1998; Yang, Scott et al. 2009).
The ultimate objective for each of these controls, independently and in combination is to maintain homeostasis during everyday demands and in the face of a homeostatic perturbation. Depending on the severity to the homeostatic threat these mechanisms may fail or become altered. Disease, weight gain, physical stress and, more commonly, psychological stress can all challenge homeostasis and the controls of ingestive behavior.

Stress as a challenge to homeostasis

Stress is a term that has been borrowed from physics and applied to the biological sciences and in this context can be defined as any real or perceived threat to homeostasis. As discussed above, the term homeostasis was coined by Cannon, but in 1956 Hans Selye, in his book “The Stress of Life”, furthered Cannon's idea by proposing the general adaptation syndrome (Selye 1956). The general adaptation syndrome postulated that a general response is mounted when a demand is placed on the body (Selye 1936; Selye 1950; Selye 1956) such that confrontation of any threat to homeostasis is met with a general response to maintain homeostasis. It is now currently accepted that the body is able to respond differently to stressors based on their type and severity and can even habituate to repeated stress, but the idea of a stress response originated with Selye.

The stress response, or general adaptation syndrome, involves a prompt activation of the sympathetic nervous system and a cascade of hormone release within the hypothalamic-pituitary adrenal (HPA) axis. Rapidly, the sympathetic nervous
A system is employed to create the well-known ‘fight-or-flight’ response by readying the organism to perform either task and affects a wide range of organ systems including the cardiovascular, respiratory, digestive and endocrine systems. Simultaneously, but slower in action, the HPA axis is activated. Each system provides mechanisms to allow the body to adapt and respond to the threat they are faced with in an attempt to maintain homeostasis.

The HPA axis is initiated by input into the paraventricular nucleus (PVN) from cortical and limbic structures. Corticotropin-releasing hormone (CRH) and arginine-vasopressin are neuropeptides which are released from the PVN to initiate the cascade of events along the HPA axis. CRH is released into the portal vasculature of the median eminence, which stimulates the secretion of adrenocorticotropic hormone (ACTH) into the systemic bloodstream. ACTH mainly acts on the adrenal gland to stimulate the synthesis and release of glucocorticoids (cortisol in humans, corticosterone in rodents). In turn, glucocorticoids supply negative feedback to the pituitary and hypothalamus to suppresses further release of ACTH.

Glucocorticoids in circulation act on two receptor types expressed throughout the periphery and brain: glucocorticoid receptors and mineralocorticoid receptors. These receptors are located in the cytosol and, once bound, dimerize and translocate to the nucleus where they identify glucocorticoid-response element sites on DNA and effect transcription of glucocorticoid-related genes.
Circulating glucocorticoids increase the level of plasma glucose through the stimulation of gluconeogenesis, glycogen degradation and inhibition of glucose uptake; they also mobilize amino acids from extrahepatic tissue, stimulate lipolysis and increase the metabolic rate. Continual high levels of glucocorticoids are beneficial in some situations, but for most, are damaging and can lead to disease (McEwen 2008). Based on this McEwen proposed the term *allostatic load* to describe the collective ‘wear and tear’ of chronically high (or low) levels of glucocorticoids exposure. The term *allostasis* was first described by Sterling and Eyer (1988) as a way to ‘maintain stability through change’ suggesting that an individual can behaviorally and physiologically adjust its response based on the current situation or state of the animal (Sterling and Eyer 1988). McEwen’s allostatic load is useful in describing the range of stress-related disease, as these often result from the effects of ‘wear and tear’ on the body following chronic exposure to glucocorticoids. One such disease is obesity.

**Stress, food intake, body weight, body composition**

Stress can increase or decrease food intake and body weight depending on the type, severity and length of the stressor. In the human population, daily stress such as school exams, public speaking, work-related issues, and interpersonal matters can lead to the increase in food intake and body weight; however stressors like traumatic grief or combat decrease food intake and body weight (Popper, Smits et al. 1989; Prigerson, Bierhals et al. 1997; Epel, Lapidus et al. 2001; Roberts, Troop et al. 2007; O'Connor, Jones et al. 2008; Nishitani, Sakakibara et al. 2009). Furthermore, sustained high
levels of cortisol are associated with the development of visceral adiposity, dislipidemia, cardiovascular disease and insulin resistance (Bjorntorp 1996; Bjorntorp 2001).

Animal models also exhibit changes in body weight and food intake following stress exposure and result in similar metabolic consequences (Ely, Dapper et al. 1997; Meerlo, Overkamp et al. 1997; Rybkin, Zhou et al. 1997; Czech, Klosterman et al. 1998; Valles, Marti et al. 2000; Bielajew, Konkle et al. 2002; Torres, Gamaro et al. 2002; Bekris, Antoniou et al. 2005; Foster, Solomon et al. 2006; Solomon, Foster et al. 2007; Coccurello, D'Amato et al. 2009). Therefore, in order to appreciate the consequences of stress exposure in the human population animal models are employed in the laboratory to gain a deeper understanding of the mechanisms and changes underlying the stress-related effects. The most common type of stress experienced on a daily basis in the human population is social stress. Social stress can manifest from interpersonal relationships, a job and family life. The most common model of social stress in the laboratory is social defeat where a resident animal is confronted with an intruder initiating aggressive interactions and resulting in the defeat of the intruder and dominance in the resident. The use of different species has produced divergent results. For example, in rodents acute defeat results in decreased food intake and body weight gain, whereas chronic defeat increases food intake (Meerlo, Overkamp et al. 1997; Coccurello, D'Amato et al. 2009); in contrast Syrian hamsters increase their food intake and body weight independent of the number of defeat episodes (Foster, Solomon et al. 2006; Solomon, Foster et al. 2007). Subordinate stress in a mouse model of social defeat leads to weight gain, whereas dominance in this paradigm induces suppression
of weight gain (Bartolomucci, Pederzani et al. 2004). Similarly, in a recent study, subordinate rhesus monkeys display increased food intake (Wilson, Fisher et al. 2008).

As discussed above, any stress-mediated change to food intake is dependent on underlying alterations to meal patterns. Restraint stress and noise exposure decrease meals size and duration (Krebs, Macht et al. 1996; Varma, Chai et al. 1999; Tabarin, Diz-Chaves et al. 2007); however, the effects of social stress on meal patterns are currently unknown.

Clinical relevance

Although the rise of obesity has finally reached a plateau after years of growth, up to 35% of adults in the United States remain obese (defined as a body mass index greater than 30) (Flegal 2005). Additionally, reports of daily stress continue to grow. In fact, a recent survey conducted by the U.S. Center for Disease Control and Prevention, reports that up to 40% of workers find their job “very or extremely stressful”, where up to 29% report they are “often stressed by their work” (Sauter, Murphy et al. 2008). It is well known that obesity and stress are associated with many complications with cardiovascular disease, endocrine and immune dysfunction, psychological disease, certain cancers, diabetes and a shortened lifespan (Bjurntorp 1996; Bjorntorp 2001; Bianchini, Kaaks et al. 2002; Bray 2004).

Lifespan can be altered by changes in meal patterns, and many of the associated complications of obesity and stress can benefit from modifications of meal frequency and/or meal size. Furthermore, it has been suggested that a reduction in meal
frequency may promote resistance to stress (Mattson, Duan et al. 2003; Mattson 2005). Therefore, understanding how a highly palatable diet and social stress can alter meal patterns is essential to the development of novel therapeutic treatments which could include ways to promote beneficial manipulations of meal patterns.

**Summary and Aims**

The studies in this dissertation explore the relationships among meal patterns, body weight and body composition under conditions of high-fat diet, chronic social stress and social subordination. It is clear that the controls of food intake, and subsequently, meal patterns can be modified by many conditions, including the consumption of a highly-palatable diet and stress exposure. Chapter 2 examines the acute exposure to high-fat diet to validate the meal pattern program established for the studies in this dissertation and to test the hypothesis that short-term high-fat diet consumption modifies body weight and alters meal patterns such that meal size predicts the gain of adiposity.

*Chapters 3-5* utilize our established rodent model of chronic social stress to examine the meal patterns during stress exposure, following stress exposure and the potential individual differences expressed within a social hierarchy. The visible burrow system (VBS) is an established ethological model of chronic social stress in which 4 males and 2 females are housed together continuously for 2-weeks. A dominance hierarchy rapidly forms among the male rats resulting in one dominant and three subordinates (however *Chapter 5* introduces another VBS phenotype and discusses its
characterization and potential implications). Specific details of the VBS have previously been described (Tamashiro, Nguyen et al. 2004; Nguyen, Tamashiro et al. 2007) and are provided in Chapter 3.

The hallmark of the VBS model is the rapid and sustained weight loss in the subordinate population as these animals show signs of severe stress including enlarged spleens, decreased testis weight, increased basal corticosterone and decreased plasma testosterone. Dominance and subordinates both exhibit thymic involution and adrenal hypertrophy suggesting that both groups experience an increased level of stress during VBS housing (Blanchard, Sakai et al. 1993; McKittrick, Blanchard et al. 1994; Blanchard, Spencer et al. 1995; Albeck, McKittrick et al. 1997; Hardy, Sottas et al. 2002; Tamashiro, Nguyen et al. 2004; Nguyen, Tamashiro et al. 2007; Tamashiro, Nguyen et al. 2007).

The VBS has been used to extensively study the metabolic effects of chronic social stress and the recovery from this exposure. As described, subordinates lose a significant amount of body weight during VBS housing. This can be attributed to the loss of adipose and lean tissue mass, whereas dominants lose adipose, but maintain lean tissue (Tamashiro 2005; Nguyen, Tamashiro et al. 2007). When placed into recovery, subordinates begin to gain weight that was lost during VBS housing; however, this weight is gained predominantly as adipose tissue and deposited preferentially in the visceral region (Tamashiro, Nguyen et al. 2007).
The overarching aim of this dissertation is to determine the food intake patterns and microstructure of this behavior to determine if the body compositional changes can be attributed to the meal taking strategy of animals exposed to VBS stress and recovery; and, to explore potential mechanisms mediating the changes in ingestive behavior.

An important point to the study of meal patterns associated with stress includes the fact that previous research examines the ingestive behavior following the stressful experience. The studies presented in Chapter 3 are the first to report meal patterns during chronic social stress as opposed to following the stress exposure. The implications for these data are significant as the previous research more correctly evaluates food intake and meal patterns in a recovery period from stress. The hypothesis of this study is that the microstructure of the reduced food intake in the subordinate population contributes to the loss of adipose and lean tissue and that NPY expression would be altered in this population identifying a potential impaired mechanism in the reduction of food intake in a state of negative energy balance.

Studies in Chapter 4 determine if any of the observed meal pattern changes reported in Chapter 3 are sustained throughout recovery from stress exposure. Subordinates have high levels of circulating glucocorticoids from VBS exposure, and these levels do not immediately clear from circulation once removed from the VBS. Therefore, if any of the ingestive behavioral changes that incurred during VBS exposure endured throughout recovery (or are altered as a result of recovery), then these
patterns, along with the potential remaining high levels of glucocorticoids or unknown molecular changes, could have great implications on body weight and composition. Chapter 4 tests the hypothesis that meal pattern behavior, expressed as an increase in meal size, induces the hyperphagia of subordinate animals during recovery and this combined with an increase in hypothalamic NPY expression contributes to the gain of adiposity.

The final chapter of this dissertation explores a novel subordinate-like phenotype within the VBS hierarchy to explore if individual differences are important in mediating the body weight, body composition and ingestive behavior consequences to chronic social stress exposure. Different coping strategies and the role of anxiety are discussed in relation to VBS stress.

Together, the data presented in this dissertation suggest that the meal taking strategies of dominant and subordinate animals largely affect their body weight and compositional changes observed during VBS stress and recovery. Future studies can now examine the potential impaired or altered controls of ingestive behavior and determine an intervention strategy to prevent the related consequences and ultimately reduce the occurrence of stress-related disease.
References


Chapter 2

Acute exposure to a high-fat diet alters meal patterns and body composition.
Introduction

Overweight and obesity are chronic global health issues in children and adults around the world (Flegal 2005; Ogden, Yanovski et al. 2007) and are associated with heart disease, diabetes, fatty liver, kidney disease, certain cancers, disability and mortality (Hjelkrem, Torres et al. 2008; Misra and Khurana 2008; Navarro-Diaz, Serra et al. 2008; Bays 2009; Fair and Montgomery 2009). The increased incidence of these health issues is often attributed to increased food intake, specifically a high-fat diet, and a decrease in energy expenditure (Gortmaker, Dietz et al. 1990; Woods, D'Alessio et al. 2004; Ogden, Yanovski et al. 2007; Judge, Zhang et al. 2008).

High-fat diet is generally palatable and affordable and, often, marketing promotes the consumption of high fat foods making them more desirable and accessible, especially in westernized nations (Drewnowski 1997; Maffeis 2000; Astrup, Dyerberg et al. 2008; Misra and Khurana 2008). Humans prefer high-fat foods to carbohydrate-rich choices leading to passive overconsumption and subsequent weight gain and increased adiposity (Blundell and MacDiarmid 1997; Ricketts 1997). Furthermore, the preference for high-fat foods increases with the level of adiposity (Mela and Sacchetti 1991) suggesting that this preference is involved in the development and maintenance of obesity. Like humans, many animals also show a preference for a high-fat diet (Castonguay, Dallman et al. 1986; Shor-Posner, Brennan et al. 1994; Alsio, Roman et al. 2008) and have similar metabolic consequences (Woods, D'Alessio et al. 2004; Buettner, Scholmerich et al. 2007; Young and Kirkland 2007). By increasing the palatability and accessibility of a high-fat diet, rats defend a higher body weight (Peck
1978) and continue to over-consume high-fat diet even when palatability and energy density are kept constant (Warwick and Weingarten 1995). This indicates that a high-fat diet has postingestive effects that contribute to overconsumption (Sclafani 2001) as well as orosensory and palatability influences, all of which contribute to increased weight gain and adiposity (Greenberg and Smith 1996; Sclafani 2004).

The way in which foods are consumed, or their pattern of consumption, has implications for body weight and composition (Nicklas, Baranowski et al. 2001). Animals genetically predisposed to obesity exhibit an increased meal size and altered feeding behavior even in the pre-obese, chow-fed state (Becker and Grinker 1977; Castonguay, Upton et al. 1982; Farley, Cook et al. 2003; Hagan, Chandler et al. 2003; Cottone, Sabino et al. 2007; Moran 2008). Meal patterns are affected by a variety of physiological and environmental factors including: food deprivation or restriction (Levitsky 1970; Blundell and Latham 1979; Larue-Achagiotis and Le Magnen 1980; Marin Bivens, Thomas et al. 1998), eating disorders (Elmore and de Castro 1991; Boggiano, Artiga et al. 2007), stress (Varma, Chai et al. 1999; Morgan, Yanovski et al. 2002), pharmacological treatments (Sakata, Fujimoto et al. 1984; Leibowitz, Alexander et al. 1993; Davoodi, Kalinichev et al. 2008), nicotine (Bellinger, Wellman et al. 2005; Wellman, Bellinger et al. 2005), exercise (Levitsky 1970; Moran 2008), social situations (de Castro and de Castro 1989; de Castro 2004), time of day (Tempel, Shor-Posner et al. 1989; Farley, Cook et al. 2003), litter size (Drewnowski, Cohen et al. 1984), macronutrients (Tempel, Shor-Posner et al. 1989; Miller, Hrupka et al. 1994; Burton-Freeman, Gietzen et al. 1997; Bensaid, Tome et al. 2003), and hormones (Lutz, Geary
Meal patterns ultimately determine total caloric intake and examining feeding behavior on a meal-to-meal basis provides insight into the microstructure of food intake, which can specifically determine the characteristics of ingestive behavior that influence changes in physiology. For example, rats over-consume high-fat diet by having larger meals and with a shorter inter-meal interval (Inter-MI), or time between meals, than chow-fed controls (Becker and Grinker 1977; Castonguay, Upton et al. 1982; Farley, Cook et al. 2003; Cottone, Sabino et al. 2007). Although this increases overall food intake, it also indicates that a high-fat diet does not have the same satiation potency or satiety influence of other macronutrients, which could ultimately result in its overconsumption (Blundell and MacDiarmid 1997). Satiation occurs during feeding and is measured by the size and length of a meal, where satiety is the state an animal is in following consumption and is indicated by the Inter-MI (Blundell and MacDiarmid 1997; Bensaid, Tome et al. 2003).

This study examines meal patterns between high-fat and chow diet over a nine-day period to evaluate fundamental differences in unmanipulated, ad lib-fed rats to determine if differences in ingestion patterns contribute to changes in body weight and composition. Studies of meal patterns and dietary manipulations have been previously examined; however, the diets have been in different forms (liquid, snacks, pellets) and multiple testing apparati have been used (Levitsky 1974; Davies 1977; Castonguay, Upton et al. 1982; Davis and Smith 1992; Smith 2000; Wellman, Bellinger et al. 2004; Zorrilla, Inoue et al. 2005). The present study examines differences in high-fat and
chow powdered diet to test the hypothesis that high-fat fed animals take larger meals and that larger meals predict the gain of adiposity.
Methods

Animals.

Ninety-day old male Long-Evans rats (Harlan; Indianapolis, IN) were individually housed in DietMax-ID monitoring cages (#45-DMCD2R, Accuscan Instruments; Columbus, OH). The animal room was temperature- and humidity-controlled on a 12-hr light:dark cycle. Animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (1996). All protocols, animal handling and treatment were approved by the Institutional Animal Care and Use Committee at the University of Cincinnati.

Animals were implanted with a subcutaneous microchip prior to VBS housing (Trovan, Electronic Identification Devices, LTD; Santa Barbara, CA), providing each animal with a unique identification number. Each DietMax-ID cage is equipped to monitor an individual animal’s food intake using a microchip-scale system. Scales are located outside of each chamber with a food cup resting on top. Food tunnels are connected to the cage and are positioned above the food cup and scale allowing the animal’s head to enter the tunnel and reach the food cup. Tunnels are activated when the animal’s head enters and breaks an infrared beam triggering the microchip reader. Microchip readers and scales are connected to a central analyzer, which records time of entry, duration of entry and changes in food cup weight. (See Figure 1)

Following habituation, animals were body weight matched and divided into two groups (N=5 per group). One remained on standard laboratory chow (5% fat, 3.46
kcals/g; Teklad Lab Animal Diets-#7012, Indianapolis, IN) that was powdered and the other group was given high-fat powdered diet (20% fat, 4.54 kcals/g; Research Diets Inc, New Brunswick, NJ); each diet was available ad libitum. Food intake was monitored for 9-days, 22-hours per day, leaving 2 hours for animal care, cage maintenance and body weight measures.
Figure 1. Schematic of the DietMax cage with ID scanner equipped tunnel. Rat places head through the tunnel, which breaks the infrared sensor and activates the microchip reader. The computer time-stamps the entry and continues reporting the microchip’s presence until it is terminated. Simultaneously, the computer time-stamps changes in scale weight.
**Body Weight and Composition.**

Body weight was recorded every-other day throughout the study. Body composition was determined before initiation and following completion of the study using the whole body NMR machine (Echo-MRI, Waco, TX). Animals were placed into a clear Plexiglas tube and NMR-scanned for less than one minute, minimizing stress to the animal. Change in adipose and lean tissue was determined by calculating the difference of the pre- and post-study measurements.

**Meal Patterns.**

Meal patterns were determined using data obtained from the DietMax-ID system extracted in text format recorded each day of the experiment. The extracted data included: the entire set of data from a single scale stored as one line per reading read at 0.1s intervals while activated, followed by the entire set of data from that scale’s associated chip reader. A computer algorithm was established to combine both sets of data such that they would be time-stamp matched creating a behavioral food intake profile for each animal. The computer program was implemented in the C++ object-oriented programming language using the Microsoft Visual.Net Integrated Development Environment, 2005. The algorithm generates doubly linked-lists of “scale events” and “microchip events”. The scale event list was stepped through, reading by reading, to find the start of each meal event and the associated time-stamp. The time-stamp was used to index the microchip event list. Due to noise in scale readings, the starting weight of the meal event was found by averaging the scale readings 5 timestamps before the microchip reading, which indicates the initiation of a potential meal event.
The ending weight of the meal event was determined in a similar manner by averaging the 5 timestamps after the microchip reading was abolished. An animal's meal size was determined as the difference between the averaged starting scale weight and the end scale weight for a bout of eating. The inter-meal interval (Inter-MI) was established as the time between microchip recordings.

**Meal Pattern Criteria.**

Meals were defined as having a consumption rate (CR: grams consumed per minute) of less than 0.50 g/min (chow) or 1.2 g/min (high-fat) as this was determined to be the maximum rate at which an intact adult male rat is able to consume powdered chow or high-fat powdered diet based on previous behavioral analysis (data not shown). Feeding events that exceeded this criterion were discarded. Feeding events were combined into a single meal if the Inter-MI was 5-min or less. Food intake was calculated by summing the size of each determined meal. Meal number was calculated after the criteria were applied to the data and includes the number of meals taken in the 22-hour testing period. Total meal duration includes the time of the entire meal event (time eating + Inter-MI if it was less than 5-min) (see Figure 2). The use of these criteria accounted for greater than 95% of the food consumed in a given testing day.

**Statistics.**

Statistical analysis was done using SigmaStat v3.1. T-tests were applied to all data and Holm-Sidak post hoc analysis was preformed where appropriate. Data was considered significant when p<0.05.
### Characteristic Definition

- **Meal**: Duration + Intra-MI
- **Total Duration**: Length of meal (duration + Intra-MI) (min)
- **Duration**: Length of feeding during meal (min)
- **Intra-MI**: Length of non-feeding behavior during meal (min)
- **Inter-MI**: Length of time from termination of meal to initiation of subsequent meal (min)

**Figure 2.** Schematic of meal pattern characteristics and definitions. Gray segments represent periods of feeding and white segments represent *intra-*meal intervals. Calculations of each characteristic included the identified sections.
Results

Exposure to high-fat diet results in weight gain and body composition changes. Animals fed the high-fat diet began to gain more weight than chow-fed controls by day 5 and the difference reached statistical significance on day 9 (p<0.04) (Figure 3). During exposure to the high-fat diet animals gained more adipose tissue than chow fed controls (p<0.001). Both groups gained lean tissue and there was no statistical difference between groups in this measure (Figure 4 A&B).

Meal patterns differed between groups depending on the type of diet. Figure 5 depicts the temporal pattern of food intake in one animal given high-fat diet (upper panel) and one animal given chow diet (lower panel).

High-fat fed animals had an average overall decreased meal frequency (Chow 15.14 ± 1.22, High-fat 11.60 ± 0.51; p<0.02) (Figure 6A). However, meal size was greater in animals consuming a high-fat diet leading to an overall increase in food intake compared to chow fed controls (Meal size: Chow 4.15 ± 0.31, High-fat 6.36 ± 0.36; p<0.02. Food intake: Chow 59.60 ± 2.85, High-fat 71.44 ± 1.37, p<0.01) (Figure 6B & 7).

Average meal duration refers to the time during the meal in which animals are actively feeding whereas total duration is the time of active feeding and the length of the intrameal interval (Intra-MI). Intra-MI is defined as those periods of time during a meal
when animals break from active eating, and Inter-MI is the time between successive meals (see Figure 2). High-fat fed animals spent less time actively feeding (p<0.02) (Figure 8A), and there were no differences in total duration, average Intra-MI, or average Inter-MI (Figure 8 B-D). Intact male Long-Evans rats are able to consume a powdered high-fat diet faster than a powdered chow diet (data not shown); in an ad lib fed state both groups spontaneously fed at a rate which is half of their maximum ability. Therefore, the average consumption rate of all meals was greater in high-fat fed animals (p<0.001) (Figure 8E). During a meal, high-fat fed rats spent, on average, approximately 60% of their time feeding whereas chow fed controls spent 80% of their time actively consuming calories (p<0.001) (Figure 8F). Taken together, animals consuming a high-fat diet consume more kcals faster, in fewer, larger bouts and in less time than chow fed controls.

Often increased food intake is implicated for the gain of adiposity. However, this study suggests that meal size has greater predictive value on changes in adiposity than overall food intake as larger meals positively correlated with a gain in adipose tissue (r=0.849, p<0.005) (Figure 9).

Not only did high-fat fed animals consume larger meals overall, but 50% of the meals they consumed are considered large (>2g, (Castonguay, Upton et al. 1982)) whereas chow fed animals consumed 50% of their meals as medium sized (1-2g) (Figure 10). Despite the increased average overall food intake and meal size, high-fat
fed animals had a significantly lower satiety ratio (Inter-MI/meal size) than chow fed controls ($p<0.04$) ($Figure 11$).

Table 1 compares meal pattern characteristics between high-fat and chow-fed animals upon initial exposure to the experimental diet (day 2) and after body weight began to differentiate (day 8). Meal pattern characteristics were similar between high-fat and chow-fed groups on day 2, but by day 8 meal frequency and size were significantly different ($p<0.03$, $p<0.02$, respectively). A significant effect of day on the latency to initiate the 1st meal of the dark phase emerged ($p<0.01$) as well as an effect of diet on meal size and Intra-MI ($p<0.01$, $p<0.04$, respectively). Furthermore, there was a significant interaction of diet and day on meal number ($p<0.04$).

Table 2 depicts overall average meal characteristics between the light and dark cycles of high-fat and chow-fed animals. During the light cycle animals consuming both diets exhibited similar feeding behavior. However, high-fat fed rats had a significantly lower satiety ratio ($p<0.03$). During the dark cycle high-fat fed animals display the previously described increase in meal size and decrease in meal duration ($p<0.001$, $p<0.01$, respectively), but also a longer Intra-MI ($p<0.04$). As expected, there was a significant effect of time on meal number, % of total number, food intake and % of total food intake ($p<0.001$), indicating that both groups displayed ingestive behavior predominantly in the dark phase. Furthermore, there was an effect of diet on meal size, meal duration and satiety ratio ($p<0.01$) similar to what was described above.
Figure 3. Percent body weight change. Animals consuming high-fat diet gained significantly more weight than chow fed controls by day 9 (Day 9 $t=2.457$, $p=0.039$; HF = high-fat diet and will be labeled as such throughout the figures in this chapter).
Figure 4. Body composition. A) Animals fed a high-fat diet put on more weight as adipose tissue than chow fed controls ($t(8)=5.275$, $p<0.001$). B) There were no differences in the amount of lean tissue gained during the study between groups ($t(8)=1.124$, $p=0.293$).
**Figure 5.** Representative histograms of food intake (kcals) in an animal fed high-fat diet and another fed chow during one 22-hour period. Gray boxes indicate dark cycle.
**Figure 6.** Meal frequency and size. A) Animals fed high-fat diet consumed fewer meals than chow fed animals ($t(7)=-2.902$, $p=0.023$). B) Meal size was greater in animals fed a high-fat diet ($t(7)=3.106$, $p=0.017$).
Figure 7. Average food intake. Animals fed high-fat diet consumed more kcals than chow fed controls ($t(7)=4.016$, $p=0.005$).
Figure 8. Meal pattern characteristics. A) Meal duration was significantly shorter in high-fat fed animals ($t(7)=-3.106$, $p=0.017$). B) There was no difference in total meal duration ($t(7)=-0.555$, $p=0.596$). C,D) There were no differences in the intrameal interval or the intermeal interval between groups (C: $t(7)=1.514$, $p=0.174$; D: $t(7)=0.951$, $p=0.373$). E) The consumption rate was significantly greater in high-fat versus chow fed animals ($t(7)=6.215$, $p<0.001$).
Figure 9. The effect of meal size and food intake on the gain of adipose tissue. A) Meal size is strongly, positively correlated with the gain of adipose tissue (r=0.849, p=0.0038). B) Food intake does not have the same influence as meal size in predicting the gain in adipose tissue (r=662, p=0.0523).
Figure 10. Average distribution of meal size. Animals consuming a chow diet consume 50% of their meals between 1-2 grams where high-fat fed animals consume 50% of their meals of a size greater than 2g.
Figure 11. Average Satiety Ratio. Animals consuming a high-fat diet have a significantly lower satiety ratio than chow fed controls (t(7)=-2.464, p=0.043).
Table 1. Meal pattern characteristics during 1\textsuperscript{st} 6hrs of the dark cycle on days 2 and 8. On day 2, high-fat fed rats showed a trend for a greater meal size ($t(7)=2.129$, $p=0.071$). On day 8 high-fat fed rats ate significantly less meals, but of a larger size than chow fed controls ($t(6)=-2.905$, $p=0.027$, $t(8)=3.121$, $p=0.014$, respectively) and showed a trend for an increased Inter-MI ($t(8)=2.136$, $p=0.065$). 2-way ANOVA analysis revealed an effect of day on the time since the last meal ($F(1, 12)=12.972$, $p=0.004$) and an effect of diet on size ($F(1, 15)=13.601$, $p=0.002$) and Intra-MI ($F(1, 15)=5.106$, $p=0.039$). There was an interaction of diet and day on meal number ($F(1, 13)=5.423$, $p=0.037$).
**Table 2.** Meal pattern characteristics in the light and dark cycle. During the light cycle, animals consuming high-fat diet had a significantly lower satiety ratio (t(5)=−3.027, p=0.029); an increase in meal size and food intake approached significance (t(7)=2.133, p=0.07, t(5)=2.338, p=0.067, respectively). During the dark cycle, high-fat fed animals had an increased meal size, decreased meal duration, and a longer Intra-MI (t(7)=5.491, p<0.001, t(7)=−3.293, p=0.013, t(7)=2.483, p=0.042, respectively). 2-way ANOVA analysis showed an effect of time on meal number, % of total number, food intake and % of total food intake (F(1, 14)=24.203, p<0.001, F(1, 14)=33.627, p<0.001, F(1, 12)=44.436, p<0.001, F(1, 12)=53.678, p<0.001. There was an effect of diet on meal size, duration and satiety ratio (F(1, 14)=16.974, p=0.001, F(1, 14)=11.12, p=0.005, F(1, 12)=11.12, p=0.006, respectively).
Discussion

The purpose of this study was to examine the feeding behavior of rats fed either a high-fat or chow powdered diet, and the effects of these diets on body weight and composition. Consistent with previous studies, animals fed a high-fat diet consumed more calories via larger, yet fewer, meals (Warwick, McGuire et al. 2000; Farley, Cook et al. 2003; Warwick, Synowski et al. 2003; Synowski, Smart et al. 2005; Donovan, Paulino et al. 2007). Furthermore, despite only an acute exposure to the high-fat diet (9 days), these animals gained more weight and adipose tissue than chow-fed controls.

Consuming a high-fat diet increases adipocyte size and number (Braun and Fabry 1969; Drewnowski, Cohen et al. 1984), increases lipid and triglyceride levels in the blood (Lairon 2008), and changes fat deposition compared to a balanced meal (Votruba, Mattison et al. 2007; Santosa and Jensen 2008). However, it may be the way in which the food is consumed that has the greatest effect on adiposity. Our study suggests that meal size significantly correlates with the gain of adiposity. Furthermore, larger meals have been correlated with increased retroperitoneal depot weight and fat cell number, and not with total food intake (Drewnowski, Cohen et al. 1984). Studies in humans indicate that overconsumption in smaller, more frequent meals can prevent hyperphagic weight gain and can reduce serum lipid and cholesterol levels (Fabry and Tepperman 1970). Therefore, meal size may be the best predictor of adiposity.
The effect of meal patterns on body composition may be exacerbated in the overweight or obese state. Animals genetically prone to obesity show spontaneous meal pattern differences from lean controls. Zucker fatty rats (inherit obesity due to an autosomal Mendelian recessive trait) and OLETF rats (with a mutated cholecystokinin-1 receptor) both exhibit hyperphagia with an increase in meal size and a decrease in meal number (Becker and Grinker 1977; Moran 2008). Furthermore, outbred DIO (diet-induced obese) rats inherently increase meal size, even in the preobese state, and partition dietary fat into storage whereas diet-resistant rats have a normal meal size and preferentially shuttle dietary fat to skeletal muscle and liver (Farley, Cook et al. 2003; Cottone, Sabino et al. 2007; Bessesen, Bull et al. 2008). Together this suggests that animals prone to obesity have altered meal patterns and that this could be an inheritable trait along with body weight and composition (de Castro 1993).

However, in the current study changes in meal patterns and body composition occurred rapidly in animals not known to be particularly prone to obesity. Upon initial exposure to the high-fat diet there was no significant difference in meal number or size compared to chow-fed animals, although the high palatability of the high-fat diet most likely contributed to a trend for an increased meal size in this group. However, by day 8 meal number was reduced and size increased on the high-fat diet suggesting that the satiety signals, which were initially intact to terminate a meal, had been altered or desensitized to subsequent high-fat meals. Ingestive behavior and specifically the meal are controlled by direct and indirect factors (Smith 2000), which can be altered under numerous circumstances. Hormones released by the gastrointestinal tract act as direct
factors, or signals, which influence meal patterns by contributing to the initiation or
termination of a meal via the central nervous system and act in a macronutrient-specific
manner. Exposure to high-fat diet has been shown to change the sensitivity of satiety
signals (Paulino, Darcel et al. 2008). Cholecystokinin (CCK) is a dietary fat-specific
satiety signal released from the I-cells in the duodenal and jejunal mucosa and reduces
meal size and duration (Gibbs, Young et al. 1973; Karhunen, Juvonen et al. 2008).
Rats maintained on an isocaloric low-fat or high-fat diet showed no differences in overall
caloric intake or body weight gain. However, when presented with a high-fat, high-
calorie test diet rats previously exposed to the isocaloric high-fat diet consumed more
calories and were unresponsive to peripheral injections of CCK (Savastano and Covasa
2005). This suggests that exposure to a high-fat diet, independent of caloric content,
impairs signaling to terminate a meal. Similarly, in the present study, animals fed a
high-fat diet have a decreased overall satiety ratio after just 9 days of high-fat food
intake, suggesting that the effects of high-fat diet exposure on the reduction or
impairment of satiety signals occurs rapidly.

Despite an overall reduced satiety ratio in high-fat fed animals, no significant
differences were found in the Inter-MI between groups. This finding has previously
been reported (Farley, Cook et al. 2003), yet the literature reports a correlation between
the size of a meal and its subsequent Inter-MI such that a larger meal, indicating greater
satiation, would be followed by a longer Inter-MI suggesting increased satiety (Thomas
and Mayer 1968; De Castro 1975; Danguir 1979). Some studies call this relationship
into question (Panksepp 1973; Castonguay, Kaiser et al. 1986; Collier, Johnson et al.

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1999). The repletion-depletion theory of food intake considers meal patterns to be under homeostatic control such that meals are initiated when energy stores are low, and terminated when these stores have been replaced thus creating an association between meal size and the subsequent Inter-MI (Le Magnen and Devos 1970; Le Magnen and Devos 1984). We have come to realize that meal initiation is not completely biologically controlled, as environmental, cultural and social factors can affect meal patterns and often override homeostatic cues (Levitsky 1970; de Castro 1988; de Castro and de Castro 1989; Blundell and Stubbs 1999; Woods and Ramsay 2000; de Castro 2004; Popkin, Duffey et al. 2005; Valassi, Scacchi et al. 2008). Meal termination, however, is largely dependent on biological signals and can be altered by diet composition (Wilding 2002; D'Alessio 2008; Karhunen, Juvonen et al. 2008; Paulino, Darcel et al. 2008).

Therefore, the current study suggests that high-fat fed animals have intact meal initiation control and satiety signals based on a reduced meal frequency and similar Inter-MI to chow fed animals, however satiation is impaired based on an increased meal size which drives the overall decrease in the satiety ratio.

High-fat fed animals spent less time consuming food, likely a result of the fact that high-fat powdered diet can be, and was, consumed at a faster rate than powdered chow diet (data not shown, (Miller, Hrupka et al. 1994). It is clear that the high-fat diet is consumed in a different manner than standard laboratory chow. Figure 5 illustrates this difference suggesting chow-fed animals consume their meals in clusters, or in close proximity to one another leaving long periods without feeding behavior, whereas high-fat fed animals eat larger meals in discrete sessions, and are not temporally linked.
The cluster-like consumption pattern along with the smaller meal size could be considered nibbling in the chow-fed group, where the large meals observed in high-fat fed animals could represent a gorging behavior. Gorging and nibbling behavior have been described in the feeding patterns of lean and obese rats (Fabry and Tepperman 1970; Becker and Grinker 1977; Castonguay, Upton et al. 1982) and animals exposed to a high-fat diet (Drewnowski, Cohen et al. 1984). In the current study high-fat fed rats behaved similarly to that of gorgers by taking half of their meals of a large size (>2g). On the other hand, 51% of the meals taken by chow-fed controls were of medium size (1-2g). Gorging and nibbling have effects on body composition, such that nibblers gain less weight and adipose tissue even when consuming the same overall calories as gorgers (Wheeler, Martin et al. 1990). Additionally, others have speculated that satiety signals respond to volume of intake, not the caloric value, which could lead to overconsumption and/or gorging of high-fat diet as more calories could be consumed before a similar volume of chow diet could be reached (Blundell and MacDiarmid 1997).

It is well established that consuming a high-fat, high-energy diet stimulates weight gain, accrualment of adipose tissue and a range of metabolic disruptions ultimately increasing the risk of developing life-altering and threatening diseases and disorders. The present data demonstrate that a high-fat diet is not consumed in the same manner as a chow diet, and others have established that high-fat diet is consumed differently than a low-fat, protein rich, carbohydrate rich, or balanced diet (Miller, Hrupka et al. 1994; Shor-Posner, Brennan et al. 1994; Blundell and MacDiarmid 1997).
1997; Burton-Freeman, Gietzen et al. 1997; Nicklas, Baranowski et al. 2001). It is important to understand the microstructure of meal patterns associated with ingestive behavior to not only elucidate the effects of digestive signals on behavior and vice versa, but to appreciate how these things can affect physiology and contribute to diseases associated with increased intake of high-fat, easily accessible foods. Ultimately, studies in which meal patterns are controlled on a high-fat diet, mimicking the feeding behavior of chow-fed animals, will help determine if consuming high-fat diet like that of chow could improve the detrimental effects of high-fat diet consumption.

In conclusion, animals fed a high-fat powdered diet have an increased meal size and a decreased meal number, and an increased meal size has predictive value on the gain of adiposity. These results are consistent with other meal pattern studies (Becker and Grinker 1977; Castonguay, Upton et al. 1982; Castonguay, Kaiser et al. 1986; Smith 2000; Zorrilla, Inoue et al. 2005; Yang, Scott et al. 2009). Therefore, the equipment and analysis used in the current study is applicable to future studies in group-housed designs.
References


Chapter 3

Chronic social stress alters food intake, meal patterns and hypothalamic NPY mRNA expression.
Introduction

Stress is known to alter food intake and body weight. In humans it has been reported that psychological stress can induce weight gain and increase food intake, but stress resulting from combat or traumatic grief results in weight loss and decreased food intake (Popper, Smits et al. 1989; Georges, Mueller et al. 1993; Prigerson, Bierhals et al. 1997; Epel, Lapidus et al. 2001; Overgaard, Gamborg et al. 2006; Roberts, Troop et al. 2007; Jacobson, Smith et al. 2009; Toyoshima, Masuoka et al. 2009). Similarly, in animal models, metabolic changes are dependent on the type, severity and length of the stressor (Torres and Nowson 2007). Acute restraint stress reduces food intake (Rybkin, Zhou et al. 1997) and chronic restraint reduces body weight gain as well (Ely, Dapper et al. 1997; Torres, Gamaro et al. 2002; Fachin, Silva et al. 2008). Likewise, immobilization stress has the same effect (Valles, Marti et al. 2000; Michel, Levin et al. 2003) and exposure to chronic mild stress, which varies the type of psychological stressor over days, reduces food intake (Bielajew, Konkle et al. 2002; Bekris, Antoniou et al. 2005). Conversely, the mild stress elicited by tail pinch induces feeding and chronic exposure leads to overeating and weight gain (Czech, Klosterman et al. 1998).

In an attempt to understand the changes associated with stress observed in the human population, social stress paradigms are utilized in the laboratory to represent the type of stress most frequently experienced in daily life (Coccurello, D'Amato et al. 2009). The most common of these paradigms is the stress resulting from social defeat following a resident-intruder interaction. In rodents, a single social defeat results in reduced food intake and reduced weight gain continuing for days following the
interaction (Meerlo, Overkamp et al. 1997). However, chronic social defeat increases food intake, but does not reverse the reduced body weight gain (Coccurello, D'Amato et al. 2009). In a mouse model of chronic social defeat animals continuously have sensory contact with one another and acute daily physical interactions resulting in dominant and subordinate animals (Bartolomucci, Sacerdote et al. 2003). Dominant animals have reduced weight gain, whereas subordinates gain more weight than controls and dominants (Bartolomucci, Pederzani et al. 2004). This divergence in weight gain occurs despite hyperphagic behavior of both groups compared to controls (Moles, Bartolomucci et al. 2006; Bartolomucci, Cabassi et al. 2009). Furthermore, when defeated intermittently, consecutively and chronically, Syrian hamsters increased their food intake and body weight compared with non-defeated controls (Foster, Solomon et al. 2006; Solomon, Foster et al. 2007). These varied results exemplify the complicated relationships among stress, food intake and weight gain.

One potential source of these divergent results may include subtle, but significant differences in caloric consumption and/or meal pattern behavior. Stress not only modifies total food intake, but can also alter meal patterns. Meal patterns ultimately determine total food intake; and examining this behavior on a meal-to-meal basis offers insight into the microstructure of food intake (Geary 2005; Zorrilla, Inoue et al. 2005), and can specifically identify those characteristics of feeding that influence changes in physiology (Nicklas, Baranowski et al. 2001). For example, consuming many small meals throughout the day decreases body relative to consuming the same caloric value in few large meals (Fabry and Tepperman 1970). Restraint stress decreases meal size
and duration and stress from surgery reduces meal frequency with an increase in meal size (Varma, Chai et al. 1999; Tabarin, Diz-Chaves et al. 2007). Furthermore, an initial reduction in food intake from repeated noise exposure results from reduced meal size and shorter duration and the reduced duration is sustained despite recovery of food intake (Krebs, Macht et al. 1996). Interestingly, each of these stressors increased the rate of eating. Few studies have examined the microstructure of food intake during social stress exposure. Bhatnagar and colleagues report reduced weight gain following repeated social stress with increased food intake, specifically during the light cycle (Bhatnagar, Vining et al. 2006). This suggests that the pattern of energy consumption is altered by social stress exposure, but specifically how is unknown.

The visible burrow system (VBS) is an established animal model of chronic social stress, which induces behavioral, endocrine, physiological and neurochemical changes (Blanchard, Cholvanich et al. 1991; Blanchard, Spencer et al. 1995; Albeck, McKittrick et al. 1997; McKittrick, Magarinos et al. 2000; Hardy, Sottas et al. 2002; Lucas, Celen et al. 2004; Tamashiro, Nguyen et al. 2004; Choi, Nguyen et al. 2006; Nguyen, Tamashiro et al. 2007). Animals are housed together in a mixed-gender colony for a period of two weeks. During this time agonistic interactions occur among the males and a dominance hierarchy rapidly forms. The stress is unpredictable and induced without investigator interference. This model is relevant to our daily lives as the stress we experience on a day-to-day basis often stems from our social environment such as the workplace or family life. Previous studies using the VBS have identified that subordinate (SUB) animals exhibit weight loss, increased basal plasma corticosterone (CORT), thymic
involution and adrenal hypertrophy (Blanchard, Spencer et al. 1995; Tamashiro, Nguyen et al. 2004). SUB animals are also hypophagic compared with dominants (DOM) and controls (CON) and lose a significant amount of adipose tissue (Tamashiro, Nguyen et al. 2004; Nguyen, Tamashiro et al. 2007; Tamashiro, Hegeman et al. 2007).

Food intake and the stress response are controlled and integrated by the central nervous system and particularly within the hypothalamus. Stress triggers the hypothalamic-pituitary-adrenal (HPA) axis, which begins with input into the paraventricular nucleus (PVN) stimulating the production and release of corticotropin releasing hormone (CRH). CRH travels via the portal circulation to the anterior pituitary inducing the release of adrenocorticotropic hormone (ACTH). ACTH travels throughout the peripheral blood stream and acts on the adrenals to release glucocorticoids (corticosterone (CORT) in rats) into the circulation. CORT feeds back on the hypothalamus, hippocampus and pituitary to reduce the further activation of the HPA axis (Herman, Figueiredo et al. 2003).

CORT and other metabolic hormones act in various areas of the hypothalamus, including the arcuate (Arc) nucleus, to modulate food intake by mediating gene expression of feeding neuropeptides implicated in controlling food intake, such as Neuropeptide Y (NPY) (Schwartz, Woods et al. 2000). NPY is a potent orexigenic agent with increased expression during times of negative energy balance (Kalra and Kalra 2004). Increased plasma CORT increases Arc NPY mRNA expression (Guillaume-Gentil, Rohner-Jeanreanaud et al. 1990; Akabayashi, Watanabe et al. 1994),
and the presence of NPY is required for CORT to induce its orexigenic properties (Mastorakos and Zapanti 2004; la Fleur 2006).

While it is clear that CORT can increase food intake, it is unknown how social stress with its increased CORT levels affects meal patterns. NPY interacts with CORT but can independently increase food intake during times of negative energy balance. SUB animals within the VBS have high circulating levels of CORT and are in negative energy balance reflected by a significant reduction in bodyweight. NPY expression increases in the Arc during negative energy balance (Kalra and Kalra 2004), and also in the dorsomedial nucleus of the hypothalamus (DMH) (Lewis, Shellard et al. 1993; Guan, Yu et al. 1998; Bi, Scott et al. 2005; Bi 2007). Increased NPY expression in the DMH is associated with an increased meal size and a slightly decreased meal number (Moran, Katz et al. 1998; Moran and Bi 2006). It has been suggested that the role of NPY in the DMH is to maintain energy homeostasis following long-term alterations in energy balance (Bi 2007), similar to what SUB animals experience in the VBS. The goal of the present study was to examine meal patterns and hypothalamic NPY mRNA expression in the Arc and DMH of animals continuously exposed to chronic social stress to test the hypothesis that SUB have altered meal patterns and brain neurochemistry compared to DOM and CON which contributes to their loss of body weight and body composition changes.
Methods

Animals.

Ninety-day old male and female Long-Evans rats (Harlan; Indianapolis, IN) were individually housed in the animal facility for 3 weeks prior to experimental testing. Animals were then placed into individual DietMax cages (#45-DMCD2R, AccuScan Instruments; Columbus, OH) for one-week to habituate to consuming powdered chow (Figure 1). During this time each animal was implanted with a subcutaneous microchip (Trovan, Electronic Identification Devicees, LTD; Santa Babara, CA) for individual identification and measurement of feeding behavior in individual- and group-housed situations. Twelve animals were randomly selected and monitored with the DietMax-ID system (AccuScan Instruments; Columbus, OH) to determine baseline food intake and meal pattern behavior. The animal room was temperature- and humidity-controlled on a 6a:6p light:dark cycle. Animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (1996). All protocols, animal handling and treatment were approved by the Institutional Animal Care and Use Committee at the University of Cincinnati.

Colonies were formed consisting of 4 males and 2 females; males in each colony were weight-matched at the start of the experiment to within 25g of each other. Controls (CON) males were weight-matched to their assigned colony and individually housed with an adult female. Colonies were run in cohorts with each cohort consisting of at least 4 colonies, with corresponding CONs. Twenty-eight CON, 26 DOM and 76 SUB are included for body weight analysis; 14 CON, 14 DOM, 40 SUB for body composition measures; 4 CON, 4 DOM, 11 SUB for plasma leptin levels; and 15 CON,
10 DOM, 30 SUB for CORT measurement. Meal pattern data include analysis from 7
DOM, 21 SUB and 6 CON pair-housed males. Hypothalamic NPY measures include 9
CON, 7 DOM, 19 SUB, which were sacrificed on the morning of Day 14 of VBS housing.
We have 12 food intake monitoring tunnels (see description below), and 8 modified VBS
set-ups; therefore, not all groups could be monitored simultaneously for meal patterns.
However, each cohort of animals and controls were run following the same protocol
throughout the experiment, and all were maintained on powered chow with the same
scale-tunnel set up (see below) and therefore there are no expected differences
between cohorts. Dominance was determined based on previously described methods
(Tamashiro, Nguyen et al. 2004; Nguyen, Tamashiro et al. 2007).

**Body Weight and Composition.**

Body weight was recorded under red light every other day for each colony.
Males were removed, weighed, and immediately replaced into the same VBS chamber.
Whole-body composition was measured using the EchoMRI whole body composition
analyzer system (Echo Medical Systems, Houston, TX). Male rats were placed into the
appropriate plastic restraint tube, inserted in the EchoMRI and scanned for
approximately 50 seconds to measure body adipose and lean tissue. Time in the
restraint tube was minimized to reduce stress. Immediately following scanning, rats
were returned to their home cage. Body composition was evaluated during the
habituation period and at the end of the 2-week VBS exposure and values are
expressed as change in tissue from these time points (VBS-habituation levels).
Food intake monitoring system and modified VBS.

Every DietMax cage is equipped to monitor individual animal’s food intake using a microchip-scale system. The same set-up has been applied to 8 VBS systems. Scales are located outside of each chamber with a food cup accessed by ID scanner equipped tunnels. These tunnels are connected to the cage and are positioned above the food cup and scale allowing the animal’s head to enter the tunnel and reach the food cup. Tunnels are activated when the animal’s head enters and breaks an infrared beam triggering the microchip reader. Microchip readers and scales are connected to a central analyzer, which records time of entry, duration of entry and changes in food cup weight. (See Figures 1 and 12)

Each colony was continuously housed for 14 days in a modified VBS (Figure 12); the original model has been previously described in detail (Tamashiro, Nguyen et al. 2004; Nguyen, Tamashiro et al. 2007). Briefly, the VBS is made of opaque black Plexiglas with a large open-field area connected to smaller chambers via a series of tunnels. The apparatus is kept in constant darkness except for the large open field (surface chamber: SFC), which is maintained on a 12:12 hour light/dark cycle. Each modified VBS is equipped with the same microchip-scale system described above. Food tunnels are connected to the outer side of the VBS where a 3-inch hole has been created to allow for the animals to insert their head to reach the food cups on each scale. All tunnels and scales are connected to a central analyzer as described above. Food is provided *ad libitum* to VBS-housed animals and CONs. Food intake was
monitored throughout VBS-housing for 22-hours a day leaving 2-hours for animal care, cage maintenance and body weight measures.

Feeding data were examined in two phases of VBS housing: hierarchy formation and hierarchy maintenance. It is well established that a dominance hierarchy forms within the first few days of VBS housing and remains stable throughout the 2-week period (from behavioral analysis, data not shown). Furthermore, body weight and feeding behavior become consistent within status groups on a day-to-day basis once the colony is stable. Using statistical techniques it was determined that the differences between days within status groups was lost following Day 6 of VBS-housing, therefore, data for feeding behavior were analyzed as overall averages for the hierarchy formation phase (Days 1-6) and hierarchy maintenance phase (Days 7-14).
Figure 12. Schematic of VBS with scale and tunnel food intake monitoring system.
Meal Patterns.

Meal patterns were determined using data obtained from the DietMax-ID system extracted in text format recorded each day for a 22-hour period (1p-11a, other 2-hours allowed for daily maintenance of animals: refilling food/water, cage changes, body weights). The extracted data included: the entire set of data from a single scale stored as one line per reading, followed by the entire set of data from that scale’s associated chip reader. A computer program was established to combine these data and ascertain a behavioral profile for each animal. The computer program was implemented in the C++ object-oriented programming language using the Microsoft Visual.Net Integrated Development Environment. The algorithm generates separate linked-lists of "scale events" and “meal events”. The meal event list was stepped through, reading by reading, to find the start of each meal and the associated time-stamp. The time-stamp was used to index the scale event list. Due to noise in scale readings, the starting weight of the meal was found by averaging the scale readings 5 timestamps before the microchip reading, which indicates the initiation of a meal. The ending weight of the meal was determined in a similar manner by averaging the 5 time stamps after the microchip reading was abolished. Meal size was defined as the difference between the determined starting scale weight and the end scale weight. Inter-meal Interval (Inter-MI) was determined as the time between microchip recordings.

Meal Pattern Criteria.

Meals were defined as having a consumption rate (grams consumed per minute) of less than 0.50g/min as this was determined to be the maximum rate an adult rat is
able to consume powdered chow based on previous behavioral analysis (data not shown). Meal events that did not reach this criterion were discarded. Meal events were combined into meals if the intermeal interval (Inter-MI), time between meals, was 5 minutes or less. Food intake was calculated by summing the size of each determined meal for each subject per day. Intrameal interval (Intra-MI) includes the time during the meal when the animal is not engaged in ingestive behavior. Meal duration (referred to in chapter 2 as total duration) includes the time of the entire meal event (time eating + Intra-MI-if was less than 300 seconds). (See Figure 2)

Habituation meal pattern characteristics were measured for 7 days, calculated for each day then averaged together for an overall habituation measure. Meal patterns were measured in VBS- and CON-housed animals each day of the 14 day housing period. Meal pattern characteristics were calculated for each animal on a daily basis, and then averaged together with animals of the same status group. Further analysis included separation into light:dark cycles, examination of feeding behavior for each VBS chamber, and an overall measure of the hierarchy formation and hierarchy maintenance phases.

**Acute restraint stress challenge.**

Animals were subjected to a restraint stress test on Day 13 of VBS housing. At 1000 hr male animals were removed from their colonies/CON-housing and immediately placed into Plexiglas restraint tubes (length 21.5cm, inner diameter 6.3cm). A small blood sample (~50ul) was quickly placed into 1.5 ml microcentrifuge tubes containing
heparin via a small tail nic. Animals remained restrained for 60 minutes, when another blood sample was collected to measure each animal’s CORT response to the acute stressor. Animals were removed from the restraint tubes and placed in individual cages for 60 minutes to recover with only water available. A final blood sample was taken at the end of the 60 minute period to determine CORT levels following recovery from the acute stressor. VBS housed animals’ basal sample was taken in the dark under red light; during the 60 minute stress period the overhead white lights were turned on; and for the 60 minute recovery period, animals experienced similar lighting to the open surface area of the VBS. CON animals blood samples were all taken in normal light conditions. Animals housed in the same room were sampled at the same time.

For leptin analysis, a separate cohort of animals was removed from their colonies/CON-housing on Day 13 and a blood sample (~250 ul) was collected via tail nic, then replaced into their colony/CON housing. All samples were kept on ice until centrifugation, plasma was then removed and stored at -20°C until analyzed.

**Plasma hormone analysis.**

Total plasma CORT and plasma leptin were measured by radioimmunoassay (RIA) using the following commercially available kits: Corticosterone DA (MP Biomedicals, LLC, Solon, OH) and Rat leptin RIA kit (Linco, St. Charles, MO).
In situ hybridization.

Animals were sacrificed following VBS by rapid decapitation. Brains were immediately removed, flash frozen and placed on ice until stored in -20°C.

Brains were coronally sectioned at 14 µm on a Leica cryostat, mounted on Fisherbrand Superfrost-Plus charged glass slides (Hampton, NH) and stored at -20°C until further analysis.

Brain sections were fixed in 4% paraformaldehyde solution, rinsed in mM KPBS, acetylated in 0.25% acetic anhydride, delipidated in chloroform and dehydrated through an ethanol series. Antisense rat NPY riboprobes were generated by in vitro transcription using $^{35}$S labeled UTP. The NPY DNA construct is a 512 bp insert into a pCR4 TOPO vector. This was linerized with the restriction enzyme PvuII and transcribed with T3 polymerase.

The 15 µl riboprobe transcription reaction was made from 2.5 µl of 1.0 µg linerized DNA fragment, 5.0 µl of $^{35}$S-UTP, 1.5 µl NTP cocktail (ATP:CTP:GTP:UTP with a ratio of 33:33:33:1), 1.0 µl 1M dithiothreito (DTT), 1.0 µl 40U/µl RNase inhibitor and 1.0 µl T3 polymerase.

Riboprobe $^{35}$S percent incorporation was determined with trichloroacetic acid (TCA) precipitation. Slides were hybridized with the NPY riboprobe (1.0 x $10^6$ cpm/50ul
buffer), combined with hybridization buffer (50% dextran sulfate, 5X hybridization stock, formamide, fish sperm (ssDNA), tRNA and DTT) and covered with glass cover-slips. Slides were then placed into hybridization chambers which were moist with 50% formamide and incubated overnight at 55°C. The following morning slides were post-treated following the removal of the coverslips beginning with a wash in 2X standard saline citrate (SCC). Next, slides were incubated in RNase A for 20 minutes at 37°C, washed numerous times in 0.2X SSC, once in 65°C 0.2X SSC for 1 hour, dehydrated through an ethanol series and air-dried.

**Image analysis**

Hybridized slides were exposed to Kodak BioMax MR film for 4-6 days and subsequently developed. Film images of brain sections were captured by digital camera. Semi-quantitative microdensitometry analysis for autoradiograph images was preformed using Scion Image (Alpha 4.0.3.2; Scion Corporation, Frederick, MD) software.

Hypothalamic brain regions were identified from the captured images of the brain tissue using Paxinos and Watson rat brain atlas (Paxinos and Watson 1998). Each identified region of interest was analyzed by subtracting the non-hybridized tissue (background) from the hybridized signal within the same brain section and data expressed as corrected gray level (CGL). Twenty-four brain sections were analyzed per region per animal. Average CGL values were calculated in series for each of the three brain regions and the highest average value was used for that individual animal. $^{14}$C
standards were developed with each film and analyzed for CGL to confirm that all measured gray levels were within the liner range of the film.

Statistics.

Statistical analysis was done using SigmaStat v3.1. Repeated measure ANOVA, 1-way ANOVA, 2-way ANOVA and paired t-tests were used where appropriate. Holm-Sidak post hoc analysis was used when differences reached significance (p<0.05). Data more than three standard deviations from the mean were discarded.
Results

Body Weight and Composition.

All colonies formed dominance hierarchies as expected and displayed the typical body weight changes associated with the VBS model (Blanchard, Spencer et al. 1995; Tamashiro, Nguyen et al. 2004) including a significant loss of weight in the SUB population and an intermediate loss by DOM animals compared to CON (p<0.001) (Figure 13). Both DOM and SUB body weights reached a plateau by Day 6 and remained consistent throughout the duration of VBS housing (p<0.001).

Both DOM and SUB lost a significant amount of adipose tissue during VBS housing compared to CON (p<0.001), however SUB also lost a significant amount of lean tissue, where DOM and CON maintained or gained lean mass (p=0.005) (Figure 14 A & B).
**Figure 13.** % Change in body weight during VBS housing. DOM and SUB lose weight during VBS housing, however SUB lose significantly more than DOM. Following D6 body weight did not significantly change within each group. Data are expressed as mean ± S.E.M. $p<0.001$ * vs. CON, $^\$ vs. DOM.
Figure 14. Change in body composition following VBS housing. A) Adipose tissue. DOM and SUB lose adipose mass during VBS housing. B) Lean tissue. SUB lost a significant amount of lean mass during VBS housing. Data are expressed as mean ± S.E.M. p<0.001 * vs. CON, $ vs. DOM.
Plasma hormone measures and acute restraint stress test.

Plasma leptin levels corresponded with the loss of adipose tissue, with a significant decrease in both DOM and SUB animals (p<0.001) (Figure 15).

Following 2 weeks of VBS housing, SUB had increased basal CORT levels compared to CON and DOM (p<0.05) (Figure 16A). In response to an acute restraint challenge at the end of burrow housing, all animals mounted a stress response and recovered to basal levels; however, DOM had an a greater CORT response than SUB (p=0.005) and DOM and SUB returned to a lower baseline than CON (p=0.003) (Figure 16B).
Figure 15. Plasma leptin. DOM and SUB had decreased plasma leptin values compared to CON. Data are expressed as mean ± S.E.M. p<0.001 * vs. CON.
Figure 16A. Basal plasma CORT. SUB had elevated basal CORT at the end of VBS housing. Data are expressed as mean ± S.E.M. p<0.05 * vs. CON, $ vs. DOM
Figure 16B. Plasma CORT in response to an acute restraint challenge. All groups responded to a 60m restraint challenge with elevated CORT levels and returned to baseline during a 60m recovery period. Data are expressed as mean ± S.E.M. p<0.05

* SUB vs. CON, $ SUB vs. DOM, ** DOM and SUB vs. CON.
Food intake meal number and meal size.

DOM and SUB immediately reduced their food intake when exposed to VBS housing ($p<0.001$). DOM recovered their food intake to CON and habituation levels once the hierarchy was stable (hierarchy maintenance phase). Caloric consumption steadily increased in SUB throughout the stress period; however, it never returned to CON levels ($p<0.01$). Therefore their food intake was suppressed throughout VBS housing compared to habituation ($p<0.001$) (*Figure 17 A&B*).

Meal frequency corresponded to the food intake results such that both DOM and SUB had a reduced meal number during the hierarchy formation phase ($p<0.001$), but only SUB animals continued to eat fewer meals throughout the hierarchy maintenance phase ($p=0.018$). Both DOM and CON consumed a similar number of meals as during the habituation period, unlike SUB who consumed fewer meals compared to pre-stress exposure ($p<0.01$) (*Figure 18 A&B*).

Only SUB displayed a reduction in meal size during VBS housing ($p<0.01$); DOM and CON consumed similar sized meals compared to each other and to the habituation period. Moreover, the decreased meal size in SUB was transient as all groups consumed similar sized meals during hierarchy maintenance (*Figure 19 A&B*).

It is important to note that CON had comparable food intake, meal frequency and meal size values to their habituation phase throughout the 2-week testing period and, DOM also had similar food intake, meal number and sizes to their habituation values.
once their colony structure had become stable (hierarchy maintenance phase). In contrast, SUB continued to have altered feeding throughout the duration of VBS housing. Initially this was accomplished through a reduced meal number and size, but food intake remained reduced throughout due mainly to a decrease in meal frequency.
**Figure 17.** Food intake during VBS housing. A) Food intake during VBS housing. Hab indicates the average consumption during the 7d habituation period prior to VBS exposure. The vertical line separates the hierarchy formation and hierarchy maintenance phase of VBS housing. (see Methods) Each will be represented on the following meal pattern graphs. CON consume values comparable to Hab. DOM and SUB are hypophagic during the initial housing period, however SUB food intake remains suppressed throughout VBS housing. B) Average food intake. DOM and SUB consume less during hierarchy formation, where SUB food intake remained decreased during hierarchy maintenance. Data are expressed as mean ± S.E.M. and all subsequent meal pattern data will be expressed the same way. $p<0.05$ * vs. CON, $ vs. DOM.
A.

![Graph showing meal number over days in VBS. The graph includes data points for CON, DOM, and SUB groups.

B.

![Bar graph showing average meal number for Hierarchy Formation and Maintenance. The graph includes data points for CON, DOM, and SUB groups.]}
**Figure 18.** Meal frequency during VBS housing. A) Meal frequency during VBS housing. DOM and SUB have a reduced meal frequency upon VBS exposure, and meal number remained suppressed in SUB. B) Average meal number. Meal frequency was reduced in DOM and SUB during the hierarchy formation phase, but only remained decreased in SUB through the duration of VBS housing. p<0.02 * vs. CON, $ vs. DOM.
**Figure 19.** Meal size during VBS housing. A) Meal size during VBS housing. SUB have a decreased meal size upon VBS housing. B) Average meal size. Meal size was reduced in SUB only during the hierarchy formation phase. $p<0.05 \ast$ vs. CON, $\$ vs. DOM.
Meal duration, Intra-MI and Inter-MI.

Meal duration was initially reduced in SUB animals compared to CON during the hierarchy formation phase; however, it was significantly increased compared to DOM during the hierarchy maintenance phase (p=0.026; p=0.042 respectively) (**Figure 20B**). Compared to the habituation phase, both DOM and SUB had a shorter meal duration during hierarchy formation, and DOM meal duration remained reduced throughout VBS housing (p<0.05) (**Figure 20A**).

Additionally, the average Intra-MI, which reflects both eating time as well as time during a meal when the animals may pause to groom or drink, was increased only during the hierarchy maintenance phase in SUB animals (p<0.001) as this measure was variable in all groups during hierarchy formation. The increased Intra-MI contributed to the increased meal duration during the hierarchy maintenance phase suggesting that SUB animals take more pauses during a meal than CON or DOM animals during social housing; however, SUB animals displayed similar Intra-MI lengths as seen during habituation, where DOM and CON animals maintain a decreased length throughout social housing (p<0.006). (**Figures 21 A&B**)

The Inter-MI, or time between meals, was considerably longer in SUB animals when first housed in the VBS. As the hierarchy became stable, SUB Inter-MI shortened, although remained longer compared to both DOM and CON (p<0.05) and compared to the habituation period (p<0.001). (**Figures 22 A&B**)
When considered together, DOM and SUB animals respond to VBS housing by altering their ingestive behavior. During the time in which animals are establishing their dominance (hierarchy formation phase), DOM animals had decreased food intake resulting from a decrease in meal number; however, no other meal pattern characteristics were disturbed, compared to CON. During hierarchy formation, SUB animals had decreased food intake due to both a decreased meal number and meal size. Additionally, SUB meal duration was decreased and Inter-MI increased compared to CON suggesting that SUB ingestive behavior is dramatically altered by hierarchy formation resulting in fewer, smaller, shorter meals. CON animals food intake, meal number and size were normal compared to their own habituation measures. However, DOM and SUB had a decreased meal duration (p<0.02), CON and DOM had a disturbed Intra-MI (p<0.01), and SUB had an increased Inter-MI compared to pre-social housing (p<0.001).

During the hierarchy maintenance phase DOM animals displayed meal patterns similar to CONs. However, SUB animals continued to have disrupted ingestive behavior. SUB food intake remained decreased, but only due to a reduced meal number, not size. Additionally, SUB had an increased meal duration, Intra-MI and Inter-MI suggesting that despite a stable hierarchy, SUB animals continued to experience adverse effects on ingestive behavior from VBS housing. This is evident in an increased satiety ratio (Inter-MI/meal size) throughout VBS housing in SUB (Figure 23). Interestingly, compared to habituation housing, both DOM and CON had reduced Intra-MI lengths (p<0.006), and SUB animals had an increased Inter-MI (p<0.001).
Figure 20. Meal duration. A) Meal duration during VBS housing. Meal duration was not different between groups on any specific day, except on Day 2. B) Average meal duration. SUB took shorter meals during the hierarchy formation period, however, once the hierarchy was stable, SUB took longer meals. p<0.05 * vs. CON, $ vs. DOM.
Figure 21. Intra-meal interval.  A) Intra-MI during VBS housing.  SUB have a longer Intra-MI.  B) Average Intra-MI.  During the hierarchy maintenance phase SUB had a longer Intra-MI compared to DOM and CON.  p<0.05 * vs. CON, $ vs. DOM.
**Figure 22.** Inter-meal interval. A) Inter-MI during VBS housing. SUB have a longer Inter-MI. B) Average Inter-MI. SUB had an overall longer Inter-MI for the duration of VBS housing. p<0.05 * vs. CON, $ vs. DOM.
Figure 23. Satiety Ratio. SUB display an increased satiety ratio throughout VBS housing. $p<0.001$ * vs. CON, $\$\$ \text{ vs. DOM.}$
Meal pattern analysis within VBS chambers.

Previous reports have described differences in the location of feeding between DOM and SUB (Tamashiro, Hegeman et al. 2007). DOM spend most of their time in the open surface (SFC) chamber, whereas SUB spend the majority of their time in the Small and Large covered chambers (Blanchard, Spencer et al. 1995; Tamashiro, Nguyen et al. 2004). Accordingly, DOM consume the majority of their food in the SFC chamber, and SUB consume calories primarily in the Small chamber ($p<0.001$) \textit{(Table 3)}. In DOM this trend is apparent immediately during the hierarchy formation phase with an increased meal frequency in the SFC chamber ($p<0.001$) \textit{(Figure 24 A&C)}. SUB consumed fewer meals in the SFC chamber during hierarchy formation; however, once the hierarchy was stable SUB consumed most of their meals in the Small chamber (hierarchy maintenance phase $p<0.001$) \textit{(Figure 24 B&D)}.

Meal size was similar in all chambers indicating that the food intake differences among chambers was solely due to increased meal frequency in the SFC chamber for DOM and the Small chamber for SUB \textit{(Table 3)}.

No differences emerged in duration or consumption rate during chamber analysis. However SUB animals had a longer Intra-MI and Inter-MI in the Small and Large chambers compared to the SFC chamber ($p<0.02$) (data not shown). These differences were expected as SUB took most of their meals in these chambers and the results correspond with the overall changes discussed above.
**Figure 24.** Chamber analysis: Meal frequency.  
A) DOM meal frequency.  DOM took most meals in the SFC chamber.  B) SUB meal frequency.  SUB took most meals in the Small chamber.  C) Average DOM meal frequency within VBS chambers.  During hierarchy formation DOM consumed fewer meals in the Small and Large chambers.  D) Average SUB meal frequency within VBS chambers.  During VBS housing SUB took most meals in the Small and Large chambers.  p<0.05 * vs. SFC, # vs. Large.
Meal Number | Meal Size (g) | Food Intake (g) 
--- | --- | --- 
DOM Small | 2.54 ± 1.00 * | 1.00 ± 0.19 | 3.20 ± 0.73 * 
DOM Large | 3.00 ± 0.86 * | 0.91 ± 0.07 | 3.22 ± 0.34 * 
DOM SFC | 6.11 ± 1.22 | 1.22 ± 0.13 | 7.77 ± 1.50 
SUB Small | 4.83 ± 0.50 *# | 1.10 ± 0.07 | 7.01 ± 0.55 *# 
SUB Large | 2.82 ± 0.47 * | 0.93 ± 0.09 | 3.98 ± 0.63 
SUB SFC | 1.19 ± 0.43 | 0.88 ± 0.12 | 2.39 ± 0.44 

**Table 3.** Meal number, meal size and food intake within the VBS chambers. DOM animals consumed fewer meals in the Small and Large chambers resulting in increased food intake in the SFC chamber. In contrast, SUB animals consumed more meals in the Small and Large chambers with an increase in food intake in the Small chamber.

Data are expressed as mean ± S.E.M. *p<0.001 vs. SFC, *# vs. Large.
Meal pattern analysis during the light and dark cycle.

As discussed, SUB and DOM consumed a fewer number of meals during the hierarchy formation phase and SUB consumed fewer meals overall. These results correspond with a reduction of meals in the dark cycle in both DOM and SUB during the hierarchy formation phase (p<0.001) as well as an overall reduced frequency in both groups in the light phase (p<0.01). Once the colony was stable, DOM consumed a similar number of meals compared to CON and to habituation. SUB continued to have a suppressed meal frequency in the dark (p<0.001). However, they displayed an increased meal frequency in the light cycle during the hierarchy maintenance phase (p<0.01), and both of these were altered from the number of meals taken during the habituation phase (p<0.01). Furthermore, both CON and DOM ate the majority of their meals during the dark (p<0.001) whereas SUB consumed equal numbers in the light and dark cycles. (Figure 25 A-D)

Meal size was increased in SUB animals during the light and decreased during the dark (p<0.01) resulting in an increase in food intake during the light cycle, and a decrease during the dark (p<0.001). Furthermore, SUB had a longer meal duration and Intra-MI in the light phase compared to DOM and CON (p=0.003, p<0.001) and in increase in Inter-MI in both the light and dark cycle (p<0.001). (Table 4)
Figure 25. Light versus Dark analysis: Meal frequency. A) Meal frequency in the light. SUB consumed significantly more meals during the light phase once the hierarchy was stable. B) Meal number in the light. DOM initially took fewer meals during the dark, but recovered to CON levels. SUB had a decreased meal frequency in the dark throughout VBS housing. C) Average meal frequency in the light. Both DOM and SUB took fewer meals during the hierarchy formation, however SUB consumed more meals during the light phase once the colony was stable. D) Average meal number in the light. DOM and SUB took fewer meals in the dark during hierarchy formation, however SUB sustained a reduced meal frequency in the dark throughout VBS housing. p<0.01 * vs. CON, $ vs. DOM.
### Table 4

Meal pattern characteristics in the light and dark cycles throughout VBS housing. SUB took larger meals in the light and smaller in the dark. CON and DOM consumed more food during the dark while SUB ate less overall. SUB had a longer meal duration and Inter-MI in the light and a longer Inter-MI overall. $P<0.05$ * vs. CON of same time, $^\$ vs. DOM of same time, $^\#$ vs. dark of same status.

<table>
<thead>
<tr>
<th></th>
<th>Size (g)</th>
<th>Food Intake (g)</th>
<th>Duration (min)</th>
<th>Inter-MI (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light</td>
<td>Dark</td>
<td>Light</td>
<td>Dark</td>
</tr>
<tr>
<td><strong>CON</strong></td>
<td>1.07 ± 0.08</td>
<td>1.36 ± 0.13</td>
<td>3.24 ± 0.4$^#$</td>
<td>15.5 ± 0.83</td>
</tr>
<tr>
<td><strong>DOM</strong></td>
<td>1.20 ± 0.10</td>
<td>1.11 ± 0.10</td>
<td>2.91 ± 0.4$^#$</td>
<td>10.4 ± 1.6$^*$</td>
</tr>
<tr>
<td><strong>SUB</strong></td>
<td>1.36 ± 0.1$^#$$^#$</td>
<td>0.87 ± 0.1$^*$</td>
<td>5.53 ± 0.4$^*$</td>
<td>5.04 ± 0.6$^*$</td>
</tr>
</tbody>
</table>
Hypothalamic NPY mRNA expression.

Following VBS housing, DOM and SUB had increased NPY mRNA expression in the Arc nucleus compared to CON (p<0.001); however, no differences emerged among any of the groups in NPY mRNA expression in the DMH (*Figure 26 A&B*).
Figure 26. Hypothalamic NPY mRNA expression following VBS housing. A) Arcuate nucleus. DOM and SUB had increased NPY mRNA expression levels in the Arc nucleus. B) Dorsomedial nucleus. NPY mRNA expression did not differ among groups. Data are expressed as mean ± S.E.M. * p<0.001 vs. CON. CGL: corrected gray level.
Figure 27. Representative photomicrographs of hypothalamic NPY expression following VBS exposure.
Discussion

Socially housed SUB from this study displayed the classic physiologic and endocrine signs of VBS stress including loss of body weight and adipose tissue, reduced plasma leptin and increased basal plasma CORT (Nguyen, Tamashiro et al. 2007; Tamashiro, Hegeman et al. 2007; Tamashiro, Nguyen et al. 2007). Previous studies began to examine the food intake of animals housed in the VBS, which led to the preliminary conclusions that the weight loss exhibited by SUB can partly be associated to the reduced caloric consumption, and that these animals may have an altered pattern of feeding compared to DOM (Tamashiro, Hegeman et al. 2007). The present study thoroughly examined the microstructure and food intake patterns of animals housed in the VBS as well as CONs to determine how social stress influences ingestive behavior, and how these potential changes contribute to the metabolic consequences of VBS exposure, and it began initial exploration of brain changes that may underlie food intake and meal pattern differences.

It is well established that dynamic behavioral and hormonal changes occur throughout the 2-week VBS housing period. Offensive and defensive behaviors are at their peak in the initial phases of hierarchy formation leading to increased interactions among males (Blanchard, Spencer et al. 1995; Blanchard, Dulloog et al. 2001; Nguyen, Tamashiro et al. 2007), and serum luteinizing hormone (LH) and testosterone (T) are elevated in DOM with increased plasma CORT in both DOM and SUB. However, by Day 7 of burrow housing DOM LH, T and CORT levels are comparable to CON, whereas SUB LH and T levels are decreased (Hardy, Sottas et al. 2002). Based on
these data, statistical analysis of current meal pattern data (see Methods) and the fact that the body weight changes held stable through-out the study beginning on Day 6, the food intake and meal pattern data were analyzed based on the hierarchy formation phase and the hierarchy maintenance phase.

During hierarchy formation, DOM reduced their food intake compared to CON. This was a result of a reduced meal frequency while these animals were establishing their place in the hierarchy. No other meal pattern characteristics were altered in DOM. Furthermore, once their dominance was established and the hierarchy was stable, DOM recovered their food intake and exhibited similar meal patterns to CON. CON maintained similar meal patterns to those of the habituation period suggesting that social housing itself does not alter meal patterns when food is available ad libitum; however, in a social situation such as the VBS, which more closely resembles a rodent’s natural environment, the formation of a dominance hierarchy transiently alters meal frequency. DOM also had a reduced meal duration and Intra-MI compared to habituation suggesting that housing in a social colony and the maintenance of dominance has lasting effects. This is unlike what occurs in some human populations as a number of social settings and environments promote increased food intake, meal size, meal duration, and probably Intra-MI. De Castro (2004) suggests that 58% of the variance in food intake is from environmental influences. For example, meal size is increased up to 44% when a meal is taken with another person due to the extended duration, and the meal size will continue to increase with the more people that are present (de Castro 1988; de Castro and de Castro 1989; De Castro 1997; de Castro
Furthermore, it has been shown that meals taken at restaurants and from fast-food locations are larger than those prepared at home and that the characteristics of a neighborhood, such as easy access to a supermarket, as well as a socioeconomic status can influence meal-taking behavior (Popkin, Duffey et al. 2005).

SUB displayed reduced food intake and altered meal patterns throughout VBS housing and did not recover to habituation values. During hierarchy formation, SUB not only consumed fewer meals, but their meals were smaller and shorter with an increased Inter-MI. During hierarchy maintenance, a time in which DOM recovered their food intake patterns, SUB continued to demonstrate signs of altered ingestive behavior including taking fewer meals of longer duration with elevated Intra-MI and Inter-MI. Together, this results in SUB having an increased satiety ratio. Satiety is a state that is reached post-prandially and involves the Inter-MI, where satiation is a state that occurs during consumption and is reflective of meal size and duration (Blundell and MacDiarmid 1997; Bensaid, Tome et al. 2003). The satiety ratio involves both states, and an increase in this measure would suggest that the animal is meeting its energy demands. In the case of the SUB, the increased value is a result of a longer Inter-MI, as their meal size is not different from other groups. This is likely representing an adaptive strategy in the SUB such that the cost of procuring food is high and therefore they initiate fewer meals, thus a longer Inter-MI, to reduce the risk of an attack from the DOM. In situations of high procurement cost animals normally compensate by taking larger meals, this was not the case in SUB as their meal size was smaller during hierarchy formation and not different from CON or DOM during the hierarchy
maintenance phase. This may also be an adaptive mechanism in that SUB may be defending a lower body weight to reduce the necessary risk of taking a meal. In fact, although SUB steadily increased their food intake throughout VBS housing, they never reached CON levels, and their body weight, although stable, remained significantly reduced.

DOM and SUB were both in negative energy balance considering both groups were maintaining their body weight at lower levels than CON, and both had lost significant amounts of adipose mass, similar to other studies (Tamashiro, Nguyen et al. 2004; Michel, Duclos et al. 2005). DOM animals were likely maintaining a lower body weight due to increased activity in the VBS, and presumably increased energy expenditure although direct measures need to be done to confirm this prediction. Others studies involving social hierarchies have indicated that DOM and SUB have different activity levels, and DOM may maintain their rank at higher energy costs (Moles, Bartolomucci et al. 2006; Bartolomucci, Cabassi et al. 2009). DOM maintain or increase their lean mass, which would support this theory.

Social stress studies in multiple species have yielded conflicting results. In rats, social defeat decreases weight gain and food intake, whereas more chronic exposure increases food intake but not enough to reverse the reduced weight gain (Meerlo, Overkamp et al. 1997; Bhatnagar, Vining et al. 2006). Socially defeated Syrian hamsters gain weight and consume more food irrespective of the number of intermittent defeats (Foster, Solomon et al. 2006; Solomon, Foster et al. 2007). Mice housed in a
social environment with limited physical interaction, but with constant sensory contact, produce dominants, which have reduced weight gain and subordinates with increased weight gain, both of which lost adipose mass (Bartolomucci, Pederzani et al. 2004; Bartolomucci, Cabassi et al. 2009). Studies in stable colonies of non-human primates report increased intake in SUB animals (Wilson, Fisher et al. 2008). In the current study, VBS SUB had decreased food intake and body weight. Although these results are partly in opposition, this maybe due to differences in the models and level of perceived stress. In social defeat models animals are returned to their home cage following the interaction, and in the mouse model conspecifics were separated each day to prevent physical contact. In the VBS model the DOM is always present and the threat of physical interaction is constant, thus the level of perceived stress to the animals may be higher leading to the negative effects on body weight and composition despite a steady increase in food intake. Both models provide valuable insight into the relation of stress and body weight, however the VBS model mimics a more natural environment and induces stress independent of investigator interference.

Others have suggested that food intake and body weight may be dissociated under chronic social stress, and body weight loss is not simply a byproduct of feeding (Coccurello, D'Amato et al. 2009). Ultimately, effects of social stress depend on the duration of stress, severity of the perceived stressor and the length of exposure (Torres and Nowson 2007). For example, subordinates in the non-human primate study consume more food than the dominants (Wilson, Fisher et al. 2008), but the colonies in which they are maintained have been stable for years, which may have allowed SUB to
develop successful coping strategies to their environment. Although VBS hierarchies are stable by the 2\textsuperscript{nd} week of housing, it is possible that VBS SUB have not mastered a similar coping strategy. Food intake was increasing in these animals and it is possible that if the colony were housed together for longer periods of time the continued stability of the hierarchy would allow SUB to recover their body weight.

Despite the potential lack of a coping strategy, the pattern in which SUB consume their food can influence their body weight and composition (Nicklas, Baranowski et al. 2001). In humans, consuming more frequent meals throughout a day prevents weight gain, even in a hyperphagic state (Fabry and Tepperman 1970) and it is known that the environment can change meal patterns in human and animal populations (de Castro and de Castro 1989; Rodgers, Ishii et al. 2002; de Castro 2004). For example, if the threat of a predator is eminent, strategies of food intake may be modified for survival (Collier and Johnson 2004; Strubbe and Woods 2004). Rats will eat quicker in exposed, novel conditions versus covered, familiar environments and will alter the meal size and frequency depending on procurement cost (Fanselow, Lester et al. 1988; Whishaw, Dringenberg et al. 1992; Mathis, Johnson et al. 1995). To our knowledge, this is the first study to examine meal patterns in a rodent model of chronic social stress; however, similar to SUB, group housed pigs eat less frequently, have longer meal duration and eat faster (Bornett, Morgan et al. 2000).

Altered meal patterns suggest that signals, which control ingestive behavior, may be impaired or overridden. Food intake and meal patterns are controlled by the central
nervous system by direct and indirect factors. Indirect factors include, among others, palatability, mood, environment and social context, and involve higher order processing in the brain. Direct factors, or signals, are released in the periphery as food travels through the digestion process and act in the brainstem and hypothalamus ultimately mediating the initiation and termination of a meal (Smith 1996; Woods, Schwartz et al. 2000; Mastorakos and Zapanti 2004). A decrease in meal frequency, as seen in the SUB population, indicates that meal initiation signals may be impaired or overridden.

Ghrelin, a peptide predominantly produced by the stomach, plays a role in meal initiation. Plasma levels of ghrelin rise prior to a meal, and decline as the meal progresses (Drazen, Vahl et al. 2006). Central and peripheral administration increases meal frequency without altering meal size (Solomon, De Fanti et al. 2005; Karhunen, Juvonen et al. 2008). Since SUB have a similar meal size to CON and DOM, SUB may lack sufficient ghrelin release, or have an impairment in ghrelin signaling. However, this is unlikely as ghrelin exerts its orexigenic action via stimulation of NPY neurons in the Arc (Kojima and Kangawa 2008; Pusztai, Sarman et al. 2008; Valassi, Scacchi et al. 2008), and NPY levels were elevated in both DOM and SUB.

Plasma leptin levels were decreased in DOM and SUB as expected due to the loss of adipose tissue in both groups. Leptin is an adiposity signal released by adipocytes in proportion to the amount of adipose tissue in the organism (Schwartz, Woods et al. 2000; Woods, Schwartz et al. 2000; Woods, Benoit et al. 2004). Leptin and ghrelin have opposing effects, as leptin inhibits NPY neurons in the Arc and reduces food intake through either a reduction in meal size and/or frequency (Hulsey,
Lu et al. 1998; Zorrilla, Inoue et al. 2005; Kawakami, Okada et al. 2008). In the current study DOM and SUB displayed decreased body weight and adipose tissue, low plasma leptin, potentially elevated ghrelin, and increased NPY expression in the Arc. Together and individually, these should all lead to the stimulation of food intake. DOM animals recovered their food intake to CON levels, however SUB maintained suppressed energy consumption throughout VBS housing while experiencing uncontrollable stress induced by the DOM. This leads to one major endocrine difference between DOM and SUB, that is, SUB had increased basal levels of CORT.

Glucocorticoids (GC) can stimulate as well as inhibit food intake. Adrenalectomy reduces food intake and body weight gain, which can be reversed with glucocorticoid replacement (Kumar and Leibowitz 1988; Kumar, Papamichael et al. 1988; la Fleur 2006; Uchoa, Sabino et al. 2009). Similarly, central infusion of dexamethasone, a synthetic glucocorticoid, increases food intake (Jeanrenaud and Rohner-Jeanrenaud 2000); however, intraperitoneal administration of dexamethasone reduces food intake (Zakrzewska, Cusin et al. 1999; Jahng, Kim et al. 2008). GC release is initiated by CRH in the hypothalamus and CRH decreases food intake (Heinrichs and Koob 1992; Zorrilla, Tache et al. 2003; Zorrilla, Reinhardt et al. 2004; Tabarin, Diz-Chaves et al. 2007). Activation of the HPA axis relies on negative feedback from CORT to suppress the expression of CRH, thus ending the stress response. Under conditions of chronic stress, CRH can be up-regulated, escaping the negative feedback of GC (Makino, Hashimoto et al. 2002). Presumably, SUB have an increase in CRH expression thus suppressing their food intake, however it has previously been shown that both DOM and
SUB have increased CRH mRNA expression in the PVN compared to CON (Albeck, McKittrick et al. 1997).

Central administration of CRH reduces body weight gain and food intake (Hotta, Shibasaki et al. 1991; Richard, Lin et al. 2002). If given directly into the PVN, CRH alters meal patterns by reducing meal frequency and increasing the Inter-MI without effecting meal size (Kochavi, Davis et al. 2001; Fekete, Inoue et al. 2007). SUB from this study had comparable reductions in body weight gain, and alterations in meal patterns; and it is known that chronic stress increases the expression of CRH in the hypothalamus (Makino, Hashimoto et al. 2002). Since food intake is ultimately controlled in the hypothalamus it is possible that CRH interacts with other feeding mechanisms in this brain region and that this interaction is impaired in SUB animals.

NPY is the most potent known orexigenic agent and is heavily expressed in the Arc following food deprivation, negative energy balance and exercise (Leibowitz and Alexander 1991; Marin Bivens, Thomas et al. 1998; Kalra and Kalra 2004; Bi, Scott et al. 2005; Torregrossa, Davis et al. 2006). GCs increase the expression of NPY thus potentiating NPY-induced feeding (Wilding, Gilbey et al. 1993; Jeanrenaud and Rohner-Jeanrenaud 2000; Konno, Yoshida et al. 2008; Shimizu, Arima et al. 2008). Furthermore, the reduced food intake and body weight resulting from adrenalectomy can only be reversed if GC and NPY are both present (Kumar and Leibowitz 1988; Kumar, Papamichael et al. 1988; Zakrzewska, Sainsbury et al. 1999; Jeanrenaud and Rohner-Jeanrenaud 2000). Therefore, NPY and GC have a positive feedback
relationship; however, NPY and CRH express a negative feedback with each other. That is, NPY neurons in the Arc activate CRH neurons in the PVN, in turn CRH activation decreases NPY mRNA in the Arc (Haas, Borgundvaag et al. 1987; Haas and George 1987; Liposits, Sievers et al. 1988; Bchini-Hooft van Huijsduijnen, Rohner-Jeanrenaud et al. 1993; Heinrichs, Menzaghi et al. 1993; Menzaghi, Heinrichs et al. 1993; Krysiak, Obuchowicz et al. 1999; Schmidt, Liebl et al. 2008).

It may be that this neural feedback loop is altered by chronic stress in SUB; meaning that despite increases in Arc NPY mRNA and presumably CRH mRNA in the PVN, this interaction is unbalanced such that CRH has a stronger inhibitory effect on NPY thus suppressing its actions. In fact, chronic administration of CRH suppresses food intake, which cannot be blocked by NPY, thus the CRH signal may be overriding the NPY drive to increase food intake (Makino, Hashimoto et al. 2002; Richard, Lin et al. 2002; Mastorakos and Zapanti 2004; Stengel, Goebel et al. 2009). One mechanism for this action may involve changes in receptor expression. NPY neurons from the Arc have direct contact with NPY receptors Y1 and Y5 on CRH neurons in the PVN and can activate these neurons thus triggering the stress response (Campbell, ffrench-Mullen et al. 2001; Dimitrov, DeJoseph et al. 2007; Kakui and Kitamura 2007). Conversely, CRH receptor subtype 1 is co-expressed with NPY neurons in the Arc, however these neurons are innervated by only a small number of CRH fibers (Campbell, Grove et al. 2003). It is possible that the negative feedback loop between CRH in the PVN and NPY neurons in the Arc may involve another ligand that would bind to CRH receptors. Urocortins, a family of endogenous ligands for CRH receptors, have fibers, which
project to Arc neurons and therefore, could play a role in the negative feedback of CRH neurons to NPY neurons in the Arc (Hauger, Risbrough et al. 2006). Future studies will have to be preformed to examine the potential role of Arc NPY and PVN CRH interactions to investigate if SUB housed in the VBS have impaired or altered communication that may play a role in their suppressed food intake despite physiologic and endocrine conditions that stimulate food intake.

DOM also had increased Arc NPY mRNA following VBS housing. This result was unexpected as DOM had similar food intake values as CON, although the elevated expression may be a result of increased activity in the DOM population as wheel running has been shown to have the same effect on Arc NPY mRNA (Bi, Scott et al. 2005). NPY expression in the DMH was not different among groups. This result is similar to other studies where Arc NPY was increased following stress and DMH NPY is unchanged (Makino, Asaba et al. 1999; Liang, Byers et al. 2007). It has been suggested that the role of NPY in the DMH maintains energy homeostasis by specifically increasing meal size following long-term alterations in energy balance (Bi and Moran 2003; Bi, Robinson et al. 2003; Bi, Chen et al. 2007). We therefore expected elevated expression in the SUB population, however, the unchanged expression of NPY in the DMH following VBS housing was not surprising as there were no differences in meal size among groups. Future studies will investigate whether the increased expression of NPY in DOM and SUB will drive food intake and if meal pattern changes will result once animals are removed from the VBS and placed into recovery.
Meal pattern alterations resulting from the current study likely represent behavioral adaptations to the VBS environment. For example, SUB took longer meals and had a longer Intra-MI, likely a result of pausing many times during a meal to gauge the risk of taking that meal; that is, if the DOM is near and threatening to interact. SUB also displayed a longer Inter-MI. In an un-stressed condition, this would represent an increase in satiety, and SUB did have an increased satiety ratio (Inter-MI/meal size), however satiety ratio in a normal laboratory setting versus a social environment perhaps does not hold the same meaning. Individually housed unmanipulated laboratory rats have free access to their chow; they do not forage or store food; and there is no procurement cost. Therefore, the satiety ratio reflects balanced energy homeostasis. In the VBS, animals must examine the risk of taking a meal meaning: from the DOM perspective if taking a meal will leave his claimed territory unguarded and available for SUB to invade; and from the SUB perspective if taking a meal will leave them vulnerable to attack by the DOM. It is important to note that SUB had access to food in all three chambers and were never restricted, and chow was provided in powdered form, so it could not be stored. If procurement cost is high, animals will normally compensate by initiating fewer meals of larger size to maintain energy balance (Collier 1985). SUB did not alter their meal size suggesting that intact satiation signals could not be overridden preventing a compensatory increase in meal size.

As expected, DOM animals consumed most meals in the SFC chamber, whereas SUB ate in the Small chamber. This is reflective of where they spent the majority of their time (Tamashiro, Nguyen et al. 2004). Unexpectedly, SUB displayed an altered
circadian pattern of food intake. Once the hierarchy was established in the VBS, SUB animals consumed more meals during the light phase and had a decreased frequency of meals throughout VBS housing in the dark. While CON and DOM overall food intake was greater in the dark, SUB consumed more food in the light phase in comparison to other groups.

Rats are nocturnal and therefore sleep during the light phase and are active during the dark, when most of their meals are taken (Bare and Cicala 1960; Siegel 1961), and, if food-deprived, rats will voluntarily recover their food intake in the dark (Lima, Hell et al. 1985). However, stress can alter the predominant nocturnal food intake and sleep patterns shifting to reduced sleep and increased food intake during the light (Kant, Pastel et al. 1995; Rybkin, Zhou et al. 1997; Varma, Chai et al. 1999).

Chronic mild stress modifies sleep patterns by increasing sleep fragmentation and episodes of rapid-eye movement (REM), leading to a less stable sleep sequence (Gronli, Murison et al. 2004). Sleep deprivation, specifically loss of REM cycles, increases food intake via an increase in overall meal number, however meal size is reduced in the dark resulting in increased energy consumption in the light phase (Elomaa 1985; Suchecki, Antunes et al. 2003; Koban and Swinson 2005). As discussed above, NPY stimulates food intake. Levels of NPY peak in the PVN prior to lights off and play a role in meal anticipation (Leal and Moreira 1997; Kalra and Kalra 2004; Drazen, Wortman et al. 2005). Furthermore, NPY has circadian rhythmicity such that Arc NPY expression is differentially expressed in the light and dark cycle (Akabayashi, Levin et al. 1994; Shimokawa, Fukuyama et al. 2003). Therefore, it may
be that SUB have a dysregulated pattern of NPY production, a shifted sleep-wake cycle and interrupted sleep sessions resulting from stress and potential lack of light exposure as their time is mostly spent in the smaller VBS chambers which are kept in constant darkness. Currently it is unknown if sleep cycles are disrupted in SUB animals leading to increased food intake during the light. Importantly, food intake and the HPA axis are closely related such that food intake can synchronize the HPA to its diurnal rhythm (Leal and Moreira 1997). SUB have increased plasma basal CORT levels, however it is not known if the changes in meal patterns affects the diurnal pattern of GC secretion.

In conclusion, VBS housing induces body weight and adipose tissue loss and changes in ingestive behavior. DOM animals initially have reduced food intake due to a decreased meal frequency, but recover all meal pattern parameters to CON levels once the hierarchy is established. SUB animals have altered meal patterns throughout VBS housing. SUB animals take fewer meals, initially of smaller size resulting in decreased food intake. SUB meals are longer in duration and have an increased Intra-MI and Inter-MI likely a result of behavioral adaptations to the VBS environment. SUB also have a disrupted circadian pattern of feeding as they take more meals of larger size during the light phase. Current mechanisms of these changes are unknown, however increased basal levels of CORT and disrupted or overridden effects of NPY may be involved.
References


Chapter 4

Recovery from chronic social stress exposure:
Altered food intake and meal patterns effect body weight and body composition.
Introduction

Stress has become a part of daily life in the westernized world and can contribute to the development of cardiovascular disease, immune dysfunction and psychological disease as well as metabolic disorders including obesity (Bjorntorp 2001; Sapolsky 2004; Dimsdale 2008; Grippo and Johnson 2009). Obesity is a concurrent epidemic effecting millions around the world and can lead to similar health complications (Kyrou and Tsigos 2007). As a result, understanding the relationship between stress and obesity is essential to potentially treat and prevent the further development of the associated co-morbidities.

Stress is known to alter food intake and body weight in the human and animal population. As a general rule, in humans, daily psychological stressors such as school exams, public speaking, job stress and ego-threatening and interpersonal situations increase food intake and body weight (Epel, Lapidus et al. 2001; Roberts, Troop et al. 2007; O'Connor, Jones et al. 2008; Nishitani, Sakakibara et al. 2009), whereas stressors not experienced on an everyday basis, such as combat and traumatic grief, decrease food intake and body weight (Popper, Smits et al. 1989; Prigerson, Bierhals et al. 1997; Jacobson, Smith et al. 2009). Animal models of social stress, such as social defeat, exhibit changes in body weight and food intake. In rodents, a single defeat episode decreases food intake and body weight, whereas daily chronic defeat increases energy consumption, but does not reverse the reduction in body weight gain (Meerlo, Overkamp et al. 1997; Coccurello, D'Amato et al. 2009). Similarly, Syrian hamsters
display increased food intake and body weight following multiple social defeat episodes (Foster, Solomon et al. 2006; Solomon, Foster et al. 2007).

Ultimately, the type, severity and length of the stressor determine the metabolic effects (Torres and Nowson 2007); nevertheless, long-term exposure to high levels of glucocorticoids can lead to the development of obesity (Bjorntorp 1996; Bjorntorp 2001). For example, in humans, the recovery of weight loss associated with chronic stress results in a net gain of abdominal adiposity (Branth, Ronquist et al. 2007), and weight gain in Syrian hamsters following social defeat is predominantly adipose tissue (Foster, Solomon et al. 2006; Solomon, Foster et al. 2007).

Similar to an increase in circulating glucocorticoid levels, the pattern of food intake can also influence body weight and composition (Nicklas, Baranowski et al. 2001). Consuming fewer, larger meals promotes the gain of adipose tissue and can increase plasma levels of triglycerides, lipids and cholesterol (Fabry, Hejda et al. 1966; Fabry and Tepperman 1970; Drewnowski, Cohen et al. 1984; Plucinski, Bruner et al. 1984; Wheeler, Martin et al. 1990; Chapelot, Marmonier et al. 2006). These effects are independent of total food intake as weight gain from caloric overconsumption can be prevented by consuming smaller, more frequent meals (Fabry, Hejda et al. 1966; Fabry and Tepperman 1970; Wheeler, Martin et al. 1990).

Portion size, snacking and meal frequency have all increased in the last 20 years along with psychosocial stress and obesity (Bray and Champagne 2005; Popkin, Duffey
et al. 2005). Each of these factors is interrelated such that stress can alter body composition and promote obesity and obesity can be limited by alterations in meal patterns and a reduction in exposure to stress. Reciprocally, stress can modify meal patterns; for example, restraint stress and chronic exposure to noise reduces meal size and duration, whereas stress from surgery increases meal size with a reduction in meal frequency (Krebs, Macht et al. 1996; Varma, Chai et al. 1999; Tabarin, Diz-Chaves et al. 2007). Few studies have examined meal patterns in any detail following a chronic stress experience, specifically that of social stress, to determine if changes in meal patterns correspond to alterations in body composition.

The visible burrow system (VBS) is an animal model of chronic social stress resulting from the formation of a hierarchy among male rats (Details of this model have been previously described (Tamashiro, Nguyen et al. 2004)). SUB display characteristics of being severely stressed, including profound weight loss, adipose and lean tissue loss, decreased thymus, spleen and testes weight, decreased plasma testosterone and insulin and increased adrenal weight and basal plasma corticosterone (Blanchard, Spencer et al. 1995; Spencer, Miller et al. 1996; Hardy, Sottas et al. 2002; Tamashiro, Nguyen et al. 2004; Tamashiro, Hegeman et al. 2007). Given the endocrine profile of SUB it is of no surprise that upon recovery from VBS exposure, SUB are hyperphagic, recover lost body weight and gain adipose tissue specifically in the visceral region (Nguyen, Tamashiro et al. 2007; Tamashiro, Nguyen et al. 2007) since negative energy balance, low plasma insulin and increased circulating glucocorticoids stimulate food intake with the latter promoting visceral adipose tissue accumulation.
glucocorticoids increase food intake is through the activation of glucocorticoid type II receptors located on Neuropeptide Y (NPY) neurons within the hypothalamus (Harfstrand, Cintra et al. 1989; Ahima and Harlan 1990; Tempel and Leibowitz 1993; Tabarin, Diz-Chaves et al. 2007). NPY is a potent stimulator of food intake, and it has a close relationship to the central mechanisms of stress circuitry and is required for corticosterone to have an orexigenic effect (Bai, Yamano et al. 1985; Chronwall, DiMaggio et al. 1985; Wilding, Gilbey et al. 1993; Kalra and Kalra 2004; Mastorakos and Zapanti 2004; la Fleur 2006). Elevated plasma corticosterone increases NPY expression in distinct nuclei within the hypothalamus including the arcuate (Arc), paraventricular (PVN) and dorsomedial (DMH) nuclei (Guillaume-Gentil, Rohner-Jeanrenaud et al. 1990; Wilding, Gilbey et al. 1993; Akabayashi, Watanabe et al. 1994). NPY in these regions has been shown to stimulate food intake via an increase in meal size and meal number (Marin Bivens, Thomas et al. 1998; Moran, Katz et al. 1998; Baird, Gray et al. 2006; Moran and Bi 2006; Bi 2007; Tiesjema, Adan et al. 2007; Yang, Scott et al. 2009).

It is unknown if meal patterns are altered in DOM or SUB animals during a recovery period from VBS housing or if hypothalamic NPY mRNA expression is different from controls in these animal populations. The following study examined meal patterns and NPY expression in the Arc and DMH during a three-week recovery period from VBS exposure to test the hypothesis that SUB take larger meals and have increased
hypothalamic NPY expression and that this combination contributes to the gain of body weight primarily as adipose tissue.
**Methods**

**Animals.**

Ninety-day old Long-Evans male rats (Harlan; Indianapolis, IN) were singly housed in the animal facility for 4 weeks. For the last week, animals were habituated to powdered chow and individually housed in DietMax cages (#45-DMCD2R, AccuScan Instruments; Columbus, OH) (Figure 1). Prior to this housing, each animal was implanted with a unique subcutaneous microchip (Trovan, Electronic Identification Devices, LTD; Santa Barbara, CA) for identification and monitoring during feeding behavior. A sub-group (N=12) of animals was randomly selected and monitored during the week of habituation for baseline food intake and meal pattern data using the DietMax-ID system (AccuScan Instruments; Columbus, OH). Animals were maintained in a temperature- and humidity-controlled room on a 12 hour light:dark cycle (lights off at 1800 hours). Animals were housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (1996). All protocols, animal handling and treatment were approved by the Institutional Animal Care and Use Committee at the University of Cincinnati.

Animals were matched for body weight, grouped into standard colonies (4 male, 2 female) and housed in the VBS for 2 weeks. Control animals (CON) were pair-housed with a female for the same amount of time. *For further details see previous chapter.* Following VBS housing, animals were individually housed in DietMax cages and recovered for 3 weeks. During this time body weight was documented every other day and food intake behavior was continuously recorded using monitoring tunnels and DietMax software (see description below). Multiple cohorts of animals were used to
assess meal patterns, body composition, stress levels, and brain neurochemistry such that no experimental assay interfered with another measure. Moreover, all cohorts and CON were subjected to the same protocol and maintained on powdered chow with the same tunnel-scale setup (see below) for the duration of the experiment such that there are no expected differences among cohorts.

At the end of Week 1 and during Week 3, body composition and basal plasma CORT were assessed in separate cohorts of animals and sacrificed either on the morning of Day 7 or Day 21. All assays were separated by at least 24 hours. Body weight analysis included: Week 1: 17 CON, 19 DOM, 40 SUB; Week 3: 14 CON, 11 DOM, 25 SUB. Body compositional measures consisted of: Week 1: 6 CON, 3 DOM, 9 SUB; Week 3: 10 CON, 7 DOM, 13 SUB. Plasma CORT analysis included: Week 1: 4 CON, 4 DOM, 12 SUB; Week 3: 6 CON, 3 DOM, 9 SUB. Meal pattern data included analysis from 6 CON, 6 DOM, 18 SUB, and brain measures included: Week 1: 6 CON, 6 DOM, 14 SUB; Week 3: 13 CON, 10 DOM, 25 SUB.

Body Weight and Composition.

Body weight measures were taken under red light every other day for each colony. Males were removed, weighed, and immediately replaced back into the VBS chamber. Whole-body composition was measured using the EchoMRI whole body composition analyzer system (Echo Medical Systems, Houston, TX). Male rats were placed into the appropriate plastic restraint tube, inserted in the EchoMRI and scanned for approximately 50 seconds to measure body adipose and lean tissue. Time in the
restraint tube was minimized to reduce stress. Immediately following scanning, rats were returned to their home cage. Body composition was evaluated at the end of the 2 week VBS exposure and following 1 and 3 weeks recovery. Values are expressed as change from these time points (1 Week-VBS; 3 Weeks-VBS).

**Food intake monitoring system.**

Each DietMax-ID cage was outfitted to monitor individual food intake using a microchip-scale system. Scales were located on the side of the cage with a food cup resting on top. Food tunnels attached to the side of the cage and are placed above the food cup and scale, allowing the animal's head to enter the tunnel and reach the food cup. Tunnels were activated when the animal's head entered and broke an infrared beam triggering the microchip reader. Microchip readers and scales were connected to a central analyzer, which recorded time of entry, duration of entry and changes in food cup weight. (See Figure 1). Food was provided ad libitum and feeding behavior was monitored for 3 weeks for 22 hours a day leaving 2 hours for animal care, cage maintenance and body weight measures.

**Meal Patterns.**

Meal patterns were determined using data obtained from the DietMax-ID system extracted in text format. See Methods of chapter 3 for further information.

**Meal Pattern Criteria.**

See chapter 3 and Figure 2 for details
Plasma corticosterone analysis.

Animals were rapidly sampled for basal CORT levels following 1 and 3 weeks of individual housing. At 1000 hours animals were removed from their cage and a small blood sample (~50 ul) was immediately taken via a small tail nic and the animal was replaced into its cage. All animals housed in the same room were sampled at the same time. Samples were kept on ice until centrifugation upon which plasma was removed and stored at -20°C until analyzed.

Total basal plasma CORT was measured by radioimmunoassay using the commercially available kit: Corticosterone DA (MP Biomedicals, LLC, Solon, OH).

In situ hybridization.

Animals were either sacrificed on the morning of Day 7 (Week 1) or Day 21 (Week 3). Details of methodology provided in chapter 3.

Statistics.

Statistical analysis was done using SigmaStat v3.1. Repeated measure ANOVA, 1-way ANOVA, 2-way ANOVA and paired t-tests were used where appropriate. Holm-Sidak post hoc analysis was used when differences reached significance (p<0.05). Data more than three standard deviations from the mean were discarded.
Results

DOM and SUB animals had previously been exposed to chronic social stress, which resulted in body weight, body composition, food intake and meal pattern changes. Although these results have been discussed previously (see previous chapter), important data from the habituation and VBS housing period are included here for reference. Habituation refers to the 1-week period prior to VBS housing when animals were singly-housed in the Dietmax-ID cages (see Figure 1) to provide baseline measures of food intake and meal patterns. The hierarchy maintenance phase of VBS housing which occurs once the colony is stable and includes the last 7 days of VBS housing (see previous chapter for further details).

Body weight and body composition.

DOM and SUB animals regained weight lost from VBS exposure. DOM recovered their body weight to CON levels after 1 week of individual recovery housing, however, although SUB animals regained some weight, they did not reach CON levels during the 3-week recovery period (p<0.001) (Figure 28).

Both DOM and SUB regained weight as lean (p<0.001) and adipose tissue; however, SUB regained more weight as adipose (p=0.03) during the 1st week of recovery. By the end of the 3rd week, both DOM and SUB had gained more lean and adipose mass than CON (p<0.001). CON and DOM continued to gain lean mass throughout the recovery period (p<0.03), whereas SUB did not gain significantly more lean tissue between week 1 and week 3 of recovery. SUB did gain significant amounts
of adipose tissue \((p<0.001)\), along with DOM \((p=0.03)\), during this time and this result was more pronounced in the SUB population.  \((Figure 29 A&B)\)

Taken together, DOM and SUB regained weight lost during stress exposure. All groups’ body composition changed during the 1st week of recovery with DOM and SUB gaining lean mass, and SUB gaining adipose tissue. By the conclusion of recovery, DOM and SUB had gained more lean and adipose mass than CON, but only the CON and DOM had gained lean mass during the latter part of recovery. In contrast, the weight that SUB gained throughout recovery was mainly adipose tissue, as these animals did not gain significantly more lean mass after the 1st week in recovery.

**Plasma Corticosterone.**

There were no statistical differences in basal plasma levels of CORT between DOM and SUB following 1 week or 3 weeks recovery \((Figure 30)\).
Figure 28. Percent body weight change during recovery. The VBS time point indicates the body weight of each group on the last day in the burrow. Both DOM and SUB regained lost body weight, however SUB animals did not reach CON levels. Data are expressed as mean ± S.E.M. * p<0.001 vs. CON; $ p<0.001 vs. DOM.
**Figure 29.** Body composition. A) DOM and SUB gained lean mass during the recovery period. CON and DOM increased their lean tissue between week 1 and week 3, where SUB did not. B) SUB began to gain more adipose tissue during the 1st week of recovery and had gained significantly more than both DOM and CON by the conclusion of the recovery period. Data are expressed as mean ± S.E.M. * p<0.05 vs. CON; $ p<0.001 vs DOM; # p<0.05 vs. Week 1 of same status.
**Figure 30.** Basal plasma corticosterone. No statistical differences were found. Data are expressed as mean ± S.E.M.
Food intake.

DOM and SUB were hyperphagic following VBS exposure compared to CON (p=0.009) (Figure 31B). CON food intake was similar to that in the habituation and hierarchy maintenance phase, whereas DOM and SUB consumed more food during recovery than during habituation or hierarchy maintenance (DOM: p=0.004, p=0.002; SUB: p=0.03, p<0.001, respectively). Both DOM and SUB had increased food intake compared to CON during weeks 1 and 3 (p=0.03, p=0.001, respectively), but DOM hyperphagia was more pronounced during week 3, and only SUB consumed more during week 2 (p=0.04) compared to CON (Figure 31A).
Figure 31. Food intake during recovery. A) Hab indicates the average consumption during the 7 day habituation period prior to VBS exposure and HM represents the average values during the hierarchy maintenance phase of VBS housing (see
Methods). The vertical lines separate the 3 weeks of the recovery period. These time points and line indicators will be represented on each of the following meal pattern graphs.

CON consumed values comparable to habituation and hierarchy maintenance values. DOM and SUB were hyperphagic during recovery compared to CON and to both the habituation and hierarchy maintenance phase. Data are expressed as mean ± S.E.M. and all subsequent meal pattern data will be expressed the same way. Week 1: ** p=0.03 SUB and DOM vs CON; Week 2: * p=0.04 SUB vs. CON; Week 3: p=0.001 ** DOM and SUB vs. CON, $ SUB vs DOM. B). Average food intake. DOM and SUB consume more food compared to CON. * p=0.009 vs. CON.
Meal patterns: Meal number and size.

Meal number during recovery was comparable to that of habituation and hierarchy maintenance in DOM and CON groups. Furthermore, meal number was not different among groups except during the final week where SUB consumed fewer meals compared to DOM (P=0.02). Despite this, SUB took more meals during recovery than during the hierarchy maintenance phase (p=0.04) (Figure 32A). Overall, there were no differences in meal number among the groups (Figure 32B).

In contrast to meal frequency, recovery meal size was increased in both DOM and SUB compared to their corresponding habituation and hierarchy maintenance values (DOM: p=0.004, p=0.002, SUB: p=0.03, p<0.001, respectively). However, only SUB had an increased meal size compared to CON during the 3 weeks of recovery (p<0.04) resulting in an overall larger meal size in SUB compared to DOM and CON (p=0.01). (Figure 33 A&B)

Overall, although both DOM and SUB both consumed more food during the recovery period compared to CON (Figure 31), it was achieved in different manners. Initially, there was an increased meal size in both groups; however, only SUB meals were significantly larger than those of CON. As the recovery period progressed, DOM had similar meal frequency and size as CON, but SUB maintained a larger meal size, and even consumed fewer meals than DOM, although the difference was not significant.
Figure 32. Meal frequency during recovery. A) Meal frequency was similar among groups during the recovery period except during the last week when SUB consumed fewer meals than DOM. $ p<0.02$ SUB vs. DOM. B) Average meal frequency. There were no significant differences in meal number during recovery.
**Figure 33.** Meal size during recovery. A) SUB animals consumed larger meals during every week of the recovery period compared to CON. B) Average meal size. Meal size was increased in SUB throughout the recovery period. * p<0.05 vs. CON.
Meal patterns: Meal duration, Intra-MI and Inter-MI.

DOM and CON sustained a consistent meal duration throughout the recovery period, and it was comparable to the length of meals taken during the habituation period. The length of meals in DOM recovered from a decreased length during hierarchy maintenance (p<0.002). SUB increased their meal duration during both week 1 and 3 (p=0.002 vs. CON, p<0.04 vs. DOM, respectively) leading to an altered meal duration as a result of stress exposure as SUB not only had an overall increase in meal duration during recovery compared to DOM (p=0.008), they also ate longer meals compared to the habituation and hierarchy maintenance phases (p=0.01, p=0.005, respectively) (Figure 34 A&B).

The Intra-MI, or time during a meal when animals may break from eating to drink or groom, was not overall different among groups during recovery (data not shown). Compared to the habituation period, all groups had a shorter Intra-MI (p<0.03); however, CON values approached the length observed during habituation with an increased length compared to the hierarchy maintenance phase (p<0.05) where DOM and SUB maintained a similar length expressed during VBS housing (data not shown).

The Inter-MI, or time between meals, was similar in DOM and CON during recovery. CON maintained consistently similar Inter-MI lengths through habituation, hierarchy maintenance and recovery, and DOM had a similar Inter-MI as in habituation relative to an increased value during hierarchy maintenance (p<0.02). SUB animals had a considerably longer Inter-MI during hierarchy maintenance, which was shorter in
recovery (p<0.001). However, SUB continued to have an increased Inter-MI compared to DOM during weeks 1 and 3 (p=0.01, p=0.009, respectively), leading to an overall increase throughout recovery (p=0.02). *(Figures 35 A&B)*

Together, following VBS exposure SUB continued to have altered meal duration, Intra-MI and Inter-MI, whereas DOM had recovered to values seen in CON and the habituation period. SUB had a longer meal duration and Inter-MI compared to DOM throughout recovery.
Figure 34. Meal duration during recovery. A) SUB animals had a longer meal duration during week 1 and 3. * p=0.002 vs. CON, $ p<0.03 vs. DOM. B) Average meal duration. SUB took longer meals in recovery compared to DOM. $ p=0.008 vs. DOM.
Figure 35. Inter-MI during recovery. A) SUB animals had a longer Inter-MI compared to DOM during week 1 and 3 of recovery. B) Average Inter-MI. Throughout recovery, SUB had a longer Inter-MI compared to DOM. $p<0.02$ vs. DOM.
Meal patterns: Light and Dark cycle.

DOM and CON consumed a similar number of meals in the dark phase throughout the recovery period and had a comparable meal frequency as they did in habituation and hierarchy maintenance. In contrast, SUB animals consumed fewer meals during the 1st week compared to both CON and DOM (p=0.008) and continued to have a reduced meal frequency compared to DOM in the last week of recovery (p=0.001). This led to an overall decrease in meal frequency in SUB during the Dark phase of recovery compared to DOM (p<0.02), however, compared to hierarchy maintenance, SUB increased their meal frequency (p<0.001) (Figure 36A). This indicates that although SUB increased their meal frequency during the dark phase from their time in the VBS, they continued to have suppressed meal frequency in recovery from VBS exposure.

SUB had an increased meal frequency compared to DOM and CON during the light phase of VBS exposure (see previous chapter) and this trend continued into the 1st week of recovery (p<0.02). SUB consumed more meals in the light cycle during the 1st week of recovery compared to habituation (p<0.03), however their meal frequency was less than in hierarchy maintenance phase (p=0.007) and was not different from CON or DOM for the remainder of recovery. CON and DOM took a similar number of meals compared to the hierarchy maintenance phase and throughout the recovery period (Figure 36B). Overall, SUB consumed fewer meals in the dark phase compared to DOM (p<0.02) and all groups consumed less meals during the light (p<0.001) (Table 5).
There were no differences in meal size among groups during the light or dark cycle, nor did time of day affect the size of meal. All groups consumed more food during the dark (p<0.001), however DOM consumed significantly more than CON and SUB consumed less than DOM (p=0.005). As discussed earlier, both DOM and SUB were hyperphagic compared to CON during recovery (Figure 31B), but their ingestive behavior was not parallel. SUB had an increased overall meal size (Figure 33B) with a slightly decreased meal number, whereas DOM had a trend for an increased meal frequency specifically during the last week of recovery (Figure 32A). Additional meal pattern differences emerged during analysis of the light and dark cycles, such that only DOM had increased food intake in the dark. Although not significant, overall increases in food intake resulted from increased number and size during the dark for DOM, and an increased number during the light and overall increase in size for SUB.

All groups took longer meals during the dark (<0.01), however SUB took longer meals when the lights were on compared to DOM (p=0.02). Furthermore, all groups expressed a longer Inter-MI during the light (p<0.001), however this was only apparent in CON as both DOM and SUB had Inter-MI lengths similar to the dark cycle (p<0.01). (Table 5)
Figure 36. Meal frequency during the dark and light phase of recovery. A) SUB animals consumed fewer meals during the dark phase than both DOM and CON during week 1, and fewer meals than DOM during week 3. B) SUB took more meals than DOM and CON in the light phase of week 1. * p<0.02 vs. CON; $ p<0.01 vs. DOM.
Table 5. Meal pattern characteristics during recovery: Light vs. Dark. Data are expressed as mean ± S.E.M. * p<0.005 vs. CON of same time; $ p<0.03 vs. DOM of same time. # p<0.01 vs. Dark.

<table>
<thead>
<tr>
<th></th>
<th>Meal Number</th>
<th>Meal Size (g)</th>
<th>Food Intake (g)</th>
<th>Meal Duration (min)</th>
<th>Inter-MI (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light</td>
<td>Dark</td>
<td>Light</td>
<td>Dark</td>
<td>Light</td>
</tr>
<tr>
<td>CON</td>
<td>2.81 ± 0.2$</td>
<td>12.8 ± 0.7</td>
<td>1.10 ± 0.1</td>
<td>1.35 ± 0.1</td>
<td>3.21 ± 0.2$</td>
</tr>
<tr>
<td>DOM</td>
<td>1.92 ± 0.1$</td>
<td>14.0 ± 1.1</td>
<td>1.40 ± 0.1</td>
<td>1.65 ± 0.1</td>
<td>2.70 ± 0.2$</td>
</tr>
<tr>
<td>SUB</td>
<td>3.14 ± 0.4$</td>
<td>10 ± 0.7$</td>
<td>1.51 ± 0.1</td>
<td>1.79 ± 0.2</td>
<td>5.53 ± 0.7$</td>
</tr>
</tbody>
</table>
Hypothalamic NPY mRNA expression.

No differences were found in NPY expression within the Arc nucleus following 1 week or 3 weeks recovery from VBS exposure (Figure 37A). DOM animals had increased NPY expression in the DMH after 1 week; however, the increase did not reach statistical significance (p=0.055). At the conclusion of the 3-week recovery period all groups had similar NPY mRNA expression in the DMH; however, SUB had increased expression compared to 1 week (Figure 37B).
Figure 37. Hypothalamic NPY mRNA expression. A) No differences were found within the Arcuate nucleus. B) DOM displayed a trend for increased NPY expression following 1 week recovery (p=0.055). SUB had increased expression at the end of 3 weeks recovery compared to just 1 week. # p<0.001 SUB vs. Week 1 of same status.
**Figure 38.** Representative photomicrographs of hypothalamic NPY expression during recovery.
**Discussion**

During the 3-week recovery period from chronic social stress DOM and SUB regained body weight lost during VBS housing and differentially altered their body composition. These results confirm previous reports (Nguyen, Tamashiro et al. 2007; Tamashiro, Nguyen et al. 2007); however, the present study further suggests that effects from chronic social stress exposure on ingestive behavior are long lasting, specifically in the SUB population, as DOM meal-taking behavior was similar to CON in frequency, size and duration. SUB consumed larger meals of greater length during recovery and took fewer meals in the dark phase.

Both DOM and SUB were hyperphagic during recovery. This result was a surprise since previous reports indicated that only SUB are hyperphagic (Tamashiro, Nguyen et al. 2007). However, the hyperphagic behavior manifests differently in SUB and DOM confirming the importance of examining meal patterns. In the DOM population there are no significant differences compared to CON in meal number or size, and it is likely that their increased overall food intake results from these animals taking more frequent meals (*Figure 32A*). Although this increase was only statistically significant during the final week of recovery, other studies have shown that even a small alteration in meal frequency such as taking one less meal a day can significantly impact body weight and composition (Chapelot, Marmonier et al. 2006). Additionally, following the first week of recovery DOM body weight was similar to CON despite remaining hyperphagic. Studies have shown that body weight can be maintained on a hypercaloric diet if meals are taken more frequently (Fabry, Hejda et al. 1966; Fabry
and Tepperman 1970). Together, these data suggest that despite being hyperphagic throughout the recovery period, the pattern of food consumption in DOM contributed to the maintenance of body weight and recovery of lean tissue.

Unlike what occurred in DOM, SUB hyperphagia resulted from an increase in meal size and a slight decrease in meal frequency. This combination of meal taking behavior leads to weight gain and adipose tissue accumulation (Fabry, Hejda et al. 1966; Fabry and Tepperman 1970; Wheeler, Martin et al. 1990; Chapelot, Marmonier et al. 2006). SUB recovered some body weight lost during VBS housing. However, despite being hyperphagic, their body weight never reached CON levels. Other studies have had similar results following stress (Bhatnagar, Vining et al. 2006; Coccurello, D'Amato et al. 2009) suggesting that food intake alone does not govern body weight (Coccurello, D'Amato et al. 2009). Furthermore, the weight gained by SUB was predominantly adipose tissue, and our lab has shown specific increases in the visceral depot (Tamashiro, Nguyen et al. 2007). Chronic stress in humans and Syrian hamsters also leads the accumulation of adipose tissue in the abdominal region (Foster, Solomon et al. 2006; Branth, Ronquist et al. 2007; Solomon, Foster et al. 2007). One contributing factor to this detrimental deposition of adipose tissue following stress is likely the pattern of food consumption. As discussed, SUB from this study consumed larger meals, but less often. In humans, reducing meal frequency by just one meal per day led to adipose tissue accumulation, and this was the case even under hypocaloric conditions (Chapelot, Marmonier et al. 2006). The combination of fewer, larger meals leads not only to obesity, but to hypercholesterolemia, diminished glucose tolerance and greater
risk for ischemic heart disease (Fabry, Hejda et al. 1966; Fabry and Tepperman 1970), complications also associated with visceral obesity (Kyrou and Tsigos 2007). Furthermore, rats that have been food restricted and allowed to recover with ad lib food availability, and likely taking larger meals, gained more weight as adipose tissue than those rats fed the same amount of food in discrete portions throughout the day (Plucinski, Bruner et al. 1984; Wheeler, Martin et al. 1990). A second contributing factor to the gain of adipose tissue in SUB may result from exposure to high levels of circulating CORT during VBS housing. Sustained long term circulating CORT leads to the development of visceral adiposity (Bjorntorp 1996; Bjorntorp 2001; Nieuwenhuizen and Rutters 2008). CORT activates lipoprotein lipase, an enzyme that promotes lipid accumulation in adipose tissue, through the stimulation of glucocorticoid receptors, which are highly expressed in visceral adipocytes (Rebuffe-Scrive, Lundholm et al. 1985; Miller, Kral et al. 1987; Bjorntorp 2001). Likewise, consuming a large meal also activates lipoprotein lipase, specifically in white adipose tissue (Fabry, Hejda et al. 1966; Fabry and Tepperman 1970; Smolin, Surh et al. 1986; Frayn, Coppack et al. 1995). It is possible that the larger meal size and exposure to high levels of glucocorticoids causes SUB to be more vulnerable to the gain of visceral adipose tissue. SUB maintained a larger meal size throughout recovery, however, following 1 week of recovery SUB basal CORT levels were comparable to those of CON. Although this suggests that elevated circulating CORT did not have an influence on the gain of adipose tissue in SUB, it is unknown if other molecular changes occurred during VBS housing which would promote visceral adipose tissue gain. It is possible that glucocorticoid receptor expression on adipocytes is altered by VBS exposure. Foot
shock stress reduces glucocorticoid receptor expression (Farias-Silva, dos Santos et al. 2004). Another possibility is that the expression of 11-\(\beta\)-hydroxysteroid dehydrogenase type 1 (11-\(\beta\)HSD1), an enzyme expressed in adipocytes which controls the local activities of glucocortioids by converting inactive 11-dehydrocorticosterone to the active form CORT, was altered during stress exposure. Adipose 11-\(\beta\)HSD1 expression is up-regulated in models of obesity and following dexamethasone treatment, and reducing this enzyme’s activity reverses many of the problems associated with obesity (Kershaw, Morton et al. 2005; Balachandran, Guan et al. 2008). Further studies will have to be done to elucidate potential molecular changes in SUB physiology to determine the complete cause of the increased adiposity, however results from this study suggest that the larger meals taken by SUB may contribute to their gain of adipose tissue following VBS exposure.

It is well known that the hypothalamus is a major site for the control of food intake. Neuropeptides expressed throughout this region have the ability to increase or decrease energy consumption. NPY is a potent stimulator of food intake and has an intricate relationship with the stress axis. Corticotropin releasing hormone, the initiator of the stress response, suppresses food intake and decreases NPY mRNA expression in the Arc (Haas, Borgundvaag et al. 1987; Haas and George 1987; Liposits, Sievers et al. 1988; Heinrichs, Menzaghi et al. 1993). Conversely, glucocorticoids, the effector hormones of the stress response, stimulate food intake and increase the expression of NPY in the Arc; and, NPY from this region can activate the HPA axis stimulating further CORT release (White 1993; Wilding, Gilbey et al. 1993; Zakrzewska, Sainsbury et al.
We examined NPY mRNA expression in the Arc and DMH after 1- and 3- weeks of recovery from VBS housing and were surprised to find no differences among any of the groups (Figure 37 & 38). Food restriction, negative energy balance and glucocorticoids all increase the expression of NPY in the Arc and DMH (Lewis, Shellard et al. 1993; Guan, Yu et al. 1998; Zakrzewska, Sainsbury et al. 1999; Jeanrenaud and Rohner-Jeanrenaud 2000; Bi, Robinson et al. 2003; Bi, Chen et al. 2007; Kinzig, Hargrave et al. 2009). Therefore it was particularly surprising that the SUB did not have elevated expression of NPY as they experienced each of these conditions while housed in the VBS. Furthermore, it has been suggested that the role of NPY in the DMH maintains energy homeostasis following long-term alterations in energy balance (Bi, Scott et al. 2004; Bi, Scott et al. 2005; Bi 2007). It is possible that NPY expression was altered during the initial days of recovery but by 1-week, when SUB were non-longer hypophagic and had begun recovering their body weight, expression had normalized to CON levels.

NPY increases food intake via an increase in meal frequency and size (White 1993; Marin Bivens, Thomas et al. 1998; Baird, Gray et al. 2006; Torregrossa, Davis et al. 2006; Tiesjema, Adan et al. 2007), and NPY in the DMH has been specifically implicated in increasing meal size with a slight decrease in frequency (Bi and Moran 2002; Yang, Scott et al. 2009). This is consistent to the SUB meal pattern behavior during recovery, yet NPY expression in the DMH was not different from DOM or CON
after 1- or 3- weeks of recovery. Together, this suggests that in our model NPY does not regulate the hyperphagia of DOM or SUB nor the increased meal size in SUB despite the close relationship of NPY and stress circuitry in the hypothalamus.

Agouti-related protein (AgRP) is another potent orexigenic peptide, which is co-expressed with 90% of the NPY neurons in the Arc (Broberger, Johansen et al. 1998; Hahn, Breininger et al. 1998). Chronic foot shock stress increases AgRP mRNA expression in the Arc and it has been suggested that NPY and AgRP are differentially regulated following stress (Helmreich, Parfitt et al. 2005; Kas, Bruijnzeel et al. 2005). Furthermore, AgRP produces its orexigenic effect through an increase in meal size (Tang-Christensen, Vrang et al. 2004; Ilnytska and Argyropoulos 2008; Santollo and Eckel 2008). Therefore, it may be that AgRP is differentially expressed in the Arc of SUB and stimulated the increase in meal size. Indeed preliminary results suggest that AgRP mRNA was increased in SUB following stress and throughout recovery compared to DOM and CON (data not shown).

SUB may also have impairments in satiation and satiety signals. Satiety signals originate from the gastrointestinal tract following the ingestion of a meal and act in the brain to reduce feeding (Smith 1996; Smith 2000; Woods, Schwartz et al. 2000; Wilding 2002; Valassi, Scacchi et al. 2008). Satiety is evaluated by the length of the Inter-MI. SUB have an increased Inter-MI compared to DOM and CON, however SUB also exhibit an increased meal size and duration indicating that meal termination signals, or satiation potency, may be altered. Many signals are known to influence meal size,
including: glucagon-like peptide-1 (GLP-1), amylin and cholecystokinin (CCK) (Woods and Seeley 2000; Ahima and Antwi 2008; D'Alessio 2008; Woods and D'Alessio 2008; Hameed, Dhillo et al. 2009). CCK is secreted from the duodenum once a meal has been initiated and was the first gut hormone identified to control food intake (Gibbs, Young et al. 1973; Liebling, Eisner et al. 1975; Moran 2004; Moran and Kinzig 2004). CCK is secreted by I-cells in the duodenal and jejunal mucosa and decreases meal size and meal duration (Kraly, Carty et al. 1978; Kissileff, Pi-Sunyer et al. 1981; Moran and Kinzig 2004; Ahima and Antwi 2008; Moran 2008; Hameed, Dhillo et al. 2009). Given that SUB have an increase in both meal size and duration it is possible that an impairment in CCK signaling or action exists in these animals. However, GLP-1, amylin and other gut factors have all been suggested to be satiety signals as they influence meal size and may all have synergistic actions with each other and/or insulin and leptin to produce satiation (Lutz, Geary et al. 1995; Lutz, Tschudy et al. 2000; Woods and Seeley 2000; Osto, Wielinga et al. 2007; Woods and D'Alessio 2008; Ruttimann, Arnold et al. 2009; Williams, Baskin et al. 2009); therefore, it is possible that any of these hormones are impaired in SUB as well. The results from the current study indicate the NPY is most likely not mediating the hyperphagia and increased meal size in recovering SUB. Further examination will have to be performed to elucidate if there is a potential increased signal driving larger meals or if there is an alteration in a satiety factor causing impaired meal termination.

SUB also exhibited an altered circadian pattern of feeding such that fewer meals were taken during the dark, when rats are most active, and an increased number of
meals were taken in the light initially following VBS exposure (Figure 36). This likely persists from the pattern observed in SUB during VBS housing since, once the hierarchy was established, SUB took most of their meals during the light (see previous chapter). Stress can alter sleep cycles, thus effecting feeding patterns often shifting to an increase in food intake during the light phase (Elomaa 1985; Kant, Pastel et al. 1995; Rybkin, Zhou et al. 1997; Varma, Chai et al. 1999; Suchecki, Antunes et al. 2003; Koban and Swinson 2005; Bhatnagar, Vining et al. 2006). Importantly, it has been suggested that large meals have different effects on body composition depending on time of day (Keim, Van Loan et al. 1997). It is currently unknown if sleep is dysregulated in SUB during recovery; however, the data suggest that behavioral alterations are sustained at least during the initial stages of recovery.

There are significant implications of this study. Stress is experienced on a daily basis and often an individual does not go through a day without experiencing some type of stressful event, likely psychological and social in nature. This suggests that we have cycles of stress and recovery throughout a day, or at least on a weekly basis. If during times following stress we consume larger, less frequent meals and have more than normal levels of GCs circulating in our blood, which have not yet cleared following our stressful episode, we have created a situation which will promote weight gain and adiposity likely in the abdominal region. In a recently established model of chronic social stress, mice were in constant sensory contact and dominance hierarchies were established with daily physical interactions (Bartolomucci, Pederzani et al. 2004). Dominants and subordinates were both hyperphagic during ‘recovery’, the time in which
they were only in sensory contact; however, dominants had decreased body weight gain and less visceral adipose tissue than subordinates. Subordinates gained more weight and had an increase in large-sized adipocytes (Bartolomucci, Cabassi et al. 2009). In that stress paradigm, animals had small ‘recovery’ periods without the threat of physical stress. It is possible that subordinate mice have similar ingestive behavior to SUB derived from the VBS in that larger meals are taken when stress is not an immediate threat, thus promoting the accrualment of adipose tissue. Based on results form the Bartolomucci paradigm, it may be important to examine the effects of variable VBS exposure, allowing animals to have intermediate recovery periods during VBS housing. This design may be most representative of the human experience in that the threat of social stress is not continuous throughout a day, but occurs in chronic episodes throughout life.

In conclusion, DOM and SUB recovered body weight lost during VBS housing and regained lean and adipose tissue. Although SUB body weight did not recover to CON levels, Sub gained significantly more adipose tissue than DOM and CON. A larger meal size and slight reduction in meal frequency taken by these animals likely contributed to their gain of adiposity however it is unknown what mechanism drives this behavior as hypothalamic NPY expression is not different among groups.
References


Chapter 5

Social stress, anxiety and Neuropeptide Y:
The OMEGA sub-phenotype in the VBS hierarchy.
Introduction

Stress can lead to changes in body weight and composition, hormonal dysregulation, interruption of sleep cycles and even neurochemical changes, and stress and particularly the dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis are also involved in many psychiatric disorders. Some of these disorders include major depression, chronic fatigue syndrome, eating disorders, alcohol abuse, and post-traumatic stress disorder (Claes 2004).

The visible burrow system (VBS) is a model of chronic social stress, which has been extensively studied in relation to behavioral, physiological, endocrine, neurochemical, and metabolic consequences of stress exposure (Blanchard, Spencer et al. 1995; Albeck, McKittrick et al. 1997; McKittrick, Magarinos et al. 2000; Blanchard, Dulloog et al. 2001; Hardy, Sottas et al. 2002; Tamashiro, Nguyen et al. 2004; Nguyen, Tamashiro et al. 2007), however, this model has never been used to specifically model psychiatric disorders. Despite this, a few findings suggest that the VBS could be a valuable tool to evaluate mental disease including: decreased dopaminergic activity within the brain reward circuitry following VBS housing (Lucas, Celen et al. 2004), decreased serotonergic binding in the hippocampus in SUB (McKittrick, Blanchard et al. 1995), increased voluntary ethanol consumption in subordinates following VBS exposure (Blanchard, Flores et al. 1992), hierarchical status shift following antidepressant treatment (McKittrick, unpublished data), and increased anxiety in subordinates during an open field test (Blanchard, Herbert et al. 1998).
Anxiety is often used synonymously with stress, but although they are related and governed by similar mechanisms, they are not the same. Stress can be defined as a real or perceived threat to homeostasis where anxiety refers more to a general response that is not a result of a specific event or stimulus, but can manifest following chronic stress (Sajdyk, Shekhar et al. 2004).

The expression of anxiety is orchestrated by the amygdala (Davis 1997; Davis 1998). Corticotropin-releasing hormone (CRH), the initiating peptide of the HPA axis, plays an anxiogenic role within the amygdala (Britton, Morgan et al. 1985; Dunn and File 1987; Baldwin, Rassnick et al. 1991). Sub-nuclei within the amygdala contain CRH receptors and CRH-expressing neurons (Inoue, Valdez et al. 2003) and psychological stress increases CRH expression and peptide levels in this region (Makino, Shibasaki et al. 1999). In contrast, Neuropeptide Y (NPY), perhaps best known for its role in food intake, has anxiolytic properties within the amygdala (Heilig, Soderpalm et al. 1989; Sajdyk, Vandergriff et al. 1999; Thorsell, Michalkiewicz et al. 2000; Kask, Harro et al. 2002). Therefore, it has been suggested that an anxiogenic role of CRH in the amygdala contributes to the overall flight-or-flight response during a stressful stimulus; however, as with many biological systems in the body, NPY acts as an opposing factor to reverse or counteract the anxiogenic effects of CRH during times in which anxiety may be maladaptive such as during chronic stress (Thorsell, Carlsson et al. 1999; Heilig 2004; Sajdyk, Shekhar et al. 2004; Eaton, Sallee et al. 2007). For example, acute restraint increases anxiety, as measured on the elevated plus maze, and amygdalar NPY levels are reduced; but, following chronic restraint where there is no longer an
The development of anxiety, as with stress resiliency and the emergence of mental disorders, varies with individual differences, leaving some individuals more vulnerable to the development of a disorder (McEwen and Stellar 1993; McEwen 2000). Some of the vulnerability depends on genetics and some on previous experience, but some causes are unknown (Zhou, Zhu et al. 2008). Preliminary data from our lab using the elevated plus maze suggest that animals who spend more time on the open arms before VBS exposure become DOM (Davis, Krause et al. 2009). However, upon closer examination it was observed that these animals became either DOM or SUB, and if SUB they seemed the most vulnerable to stress due to severe loss of body weight (Melhorn, Scott et al. 2008). This led us to consider the SUB population resulting from VBS hierarchy formation to determine if differences may exist among this status group. SUB have previously been sub-classified based on their endocrine response to a novel restraint stress test (Blanchard, Sakai et al. 1993). In contrast, we wanted to explore potential behavioral differences during VBS housing which could uncover another phenotype resulting from social subordination. We observed agonistic interactions, time spent in the open surface chamber, wounding patterns as well as a basic assessment of overall activity. Together these results led us to establish the OMEGA phenotype; that is the SUB animal that seems most vulnerable to the effects of chronic social stress.
This study explored the OMEGA phenotype as determined by behavior expressed in the VBS, and evaluated the body weight, body composition, meal patterns and stress responses to an acute restraint challenge to determine the differences between OMEGA and SUB, as well as DOM and CON. Furthermore, we test the hypothesis that OMEGA have altered amygdalar NPY expression which effects behavior during VBS housing and contributes to their OMEGA phenotype.
Methods

Animals utilized in this study were subsections of the experiments discussed in chapters 3 and 4. Therefore, many of the methods have been described in those sections including: animals used, their care and maintenance and DietMax cage housing prior to VBS exposure, the food intake monitoring system, meal patterns and meal pattern criteria and plasma corticosterone analysis. Specific methodological details unique to this chapter are provided below; please refer to previous chapters for further information.

Animals

All animals were pretested on the elevated plus maze (EPM) prior to VBS housing. Animals, which went out to one or both arms of the EPM, were identified as test subjects and placed into a colony. Three other males were body-weight matched to that animal to form standard VBS colonies of 4 males and 2 females. Controls consisted of 4 males, which went to the end of the open arm of the EPM, and 4 that displayed behavior similar to the rest of the males tested. Additional animals that were identified as potential test animals with the EPM and not chosen as a CON or colony member were not used in the experiment.

Elevated plus maze (EPM)

Prior to VBS housing animals were evaluated on the EPM. The EPM was constructed out of black Plexiglas and situated 50 cm above the floor with 2 open arms and 2 closed arms. Each animal was placed in the center of the EPM and observed for
5 minutes. Animals that explored to one or both ends of the open arms were identified and assigned to a colony or control group as described.

**Experimental timeline**

Animals were observed for 1 week in individual DietMax cages to establish habituation meal pattern behaviors. The EPM testing was performed prior to food intake monitoring. Colonies were established and animals were housed in the VBS for 2 weeks. This was followed by 3 weeks of recovery housing. Throughout the entire period food intake was recorded for the measurement of meal patterns.

Body weight was evaluated every other day throughout the entire experiment, and a body composition measure was taken using the EchoMRI (Echo Medical Systems, Houston, TX) during habituation, immediately following VBS housing, and after 1 and 3 weeks of recovery. Body composition data are expressed as change in absolute fat and lean tissue. This was accomplished by subtracting the previous transition time-point from the one reported (i.e. VBS=VBS-habituation, 1 week=1 week recovery-VBS, 3 weeks=3 weeks recovery-VBS). An acute restraint challenge was administered on the morning of day 13 of VBS housing, and following 3 weeks of recovery. A separate cohort of animals was subjected to a forced swim test either immediately following VBS or during the last week of recovery.
Forced swim test (FST)

A forced swim test was performed on a cohort of animals immediately following VBS exposure, and another during the last week of recovery (n=4 colonies per cohort). FST chambers were constructed out of Plexiglas and measured 46 cm high, 21 cm wide. Each chamber was surrounded by opaque material so each rat could not observe another’s behavior. Water temperature was between 33 and 37°C. Animals were placed 4 at a time into individual FST chambers and video-recorded for 15 minutes.

The cohort that was tested immediately following VBS housing was removed, colony-by colony, on day 14 and taken to another room where 4 FST chambers were set up. All animals were simultaneously placed into a chamber and left for 15 minutes. Once complete, all animals were removed and placed in individual cages and moved to a separate room for the completion of the recovery period. This process was repeated with the next colony of animals until all four colonies were evaluated. A separate cohort of animals was evaluated in the FST during the last week of recovery. Again, animals were brought to a separate room in groups of four and following 15 minutes of evaluation, were returned to their home cage.

The same cohort of animals was not subjected to an acute restraint stress test and the FST at the same time-point so as to not have potentially confounding data.
Behavioral analysis used to determine the OMEGA phenotype

During VBS housing, behavior was digitally recorded for 8 colonies using an infrared light source and camera mounted above each VBS apparatus. Recordings were taken for the first 6 hours of lights out (1800 hr) every other day through the 2 weeks of VBS housing.

Videos were evaluated for the first 15 minutes of every hour for each of the 6 hours of each day recorded and analyzed for specific offensive (biting, lateral attack, on-top-of, chasing), defensive (on-the-back and flight) and other behaviors such as tumbling (Blanchard, Spencer et al. 1995; Tamashiro, Nguyen et al. 2004; Nguyen, Tamashiro et al. 2007). Time spent in the open surface area as well as the location of each animal throughout the analyzed video segments was noted.

Wound counts were performed during body weight assessment every other day of VBS housing. An animal would be removed from the colony, quickly weighed and wounds from the head, back, lower back and tail were counted. The next male was removed from the colony and evaluated in the same manner. This was done two more times until all 4 males were removed, then all were replaced into the VBS before moving on to the next colony of animals. This was all done under red light, and animals were out of their colonies for 5-10 minutes.

During analysis of the wound counts it was observed that within any given colony the amount of wounding was quite variable. To correct for this we expressed the data
as a percent of the total wounds received in that colony (the sum of all four males total wounds) and expressed for the head region and body region (back + lower back).

Following the behavioral analysis it was determined that body weight loss, percent of total wounds and loss of lean mass consistently predicted OMEGA status. Based on this we were able to identify OMEGA animals from other cohorts and utilize their brain tissue for evaluation of NPY mRNA expression, along with animals identified with the described method to get measurements at the VBS and 3-week recovery time-points.


In situ hybridization

Animals were sacrificed following VBS and recovery by rapid decapitation. Brains were immediately removed, flash frozen and placed on ice until stored in -20°C.
Brains were coronally sectioned at 14 µm on a Leica cryostat, mounted on Fisherbrand Superfrost-Plus charged glass slides (Hampton, NH) and stored at -20°C until further analysis.

Brain sections were fixed in 4% paraformaldehyde solution, rinsed in mM KPBS, acetylated in 0.25% acetic anhydride, delipidated in chloroform and dehydrated through an ethanol series. Antisense rat NPY riboprobes were generated by in vitro transcription using $^{35}$S labeled UTP. The NPY DNA construct is a 512 bp insert into a pCR4 TOPO vector. This was linerized with the restriction enzyme Pvull and transcribed with T3 polymerase.

The 15 µl riboprobe transcription reaction was made from 2.5 µl of 1.0 µg linerized DNA fragment, 5.0 µl of $^{35}$S-UTP, 1.5 µl NTP cocktail (ATP:CTP:GTP:UTP with a ratio of 33:33:33:1), 1.0 µl 1M dithiothreito (DTT), 1.0 µl 40U/µl RNase inhibitor and 1.0 µl T3 polymerase.

Riboprobe $^{35}$S percent incorporation was determined with trichloroacetic acid (TCA precipitation. Slides were hybridized with the NPY riboprobe (1.0 x $10^6$ cpm/50ul buffer), combined with hybridization buffer (50% dextran sulfate, 5X hybridization stock, formamide, fish sperm (ssDNA), tRNA and DTT) and covered with glass cover-slips. Slides were then placed into hybridization chambers which were moist with 50% formamide and incubated overnight at 55°C. The following morning slides were post-
treated following the removal of the coverslips beginning with a wash in 2X standard saline citrate (SCC). Next, slides were incubated in RNase A for 20 minutes at 37°C, washed numerous times in 0.2X SSC, once in 65°C 0.2X SSC for 1 hour, dehydrated through an ethanol series and air-dried.

**Image analysis**

Hybridized slides were exposed to Kodak BioMax MR film for 4-6 days and subsequently developed. Film images of brain sections were captured by digital camera. Semi-quantitative microdensitometry analysis for autoradiograph images was preformed using Scion Image (Alpha 4.0.3.2; Scion Corporation, Frederick, MD) software.

Hypothalamic brain regions were identified from the captured images of the brain tissue using Paxinos and Watson rat brain atlas (Paxinos and Watson 1998). Amygdalar nuclei were further identified using a thionin stain. The riboprobe treated slides were soaked in Citrisolv for 5 minutes, 100% ethanol for 2 minutes and Citrisolv again for 3 minutes, then hydrated through an abbreviated ethanol series (100%, 95% and 70%) followed by purified water. Slides were dipped in thionin stain for up to 2 minutes, dehydrated and set in Citrisolv until cover-slipped. Slides were cover-slipped using Permount and laid out to dry.

Each stained section was over-laid on top of the developed film and an image was captured displaying the mRNA message and tissue fibers. Without moving the film,
a second image was captured of just the X-ray film in the same orientation. Using anatomical markers, and the Paxinos and Watson rat brain atlas (Paxinos and Watson 1998), the amygdala was analyzed for NPY mRNA expression in the central, medial and basolateral nuclei.

Each identified region of interest was analyzed by subtracting the non-hybridized tissue (background) from the hybridized signal within the same brain section and data expressed as corrected gray level (CGL). Twenty-four brain sections were analyzed per region per animal. Average CGL values were calculated in series for each of the three brain regions and the highest average value was used for that individual animal. ¹⁴C standards were developed with each film and analyzed for CGL to confirm that all measured gray levels were within the liner range of the film.

Statistics

Data are expressed as mean ± S.E.M. Statistical analysis was done using SigmaStat v3.1. Repeated measure ANOVA, 1-way ANOVA, 2-way ANOVA and paired t-tests were used where appropriate. Holm-Sidak post hoc analysis was used when differences reached significance (p<0.05). Data more than three standard deviations from the mean were discarded.
Results

As previously reported (chapters 3 and 4), all colonies formed hierarchies as expected and displayed the classic signs of VBS stress including: loss of body weight and increased basal plasma corticosterone.

Determination of the OMEGA phenotype

Of the animals identified prior to VBS housing as those that went out to one or both arms of the EPM, 4 became DOM, 2 SUB, and 2 OMEGA (data not shown) suggesting that this pretest is not a good predictor of VBS status.

Behavior was analyzed using the video recordings taken for the 1st 6 hours of lights out every other day of VBS housing. There were subtle differences among the SUB population. The most striking behavior observed was the activity of a sub-set of this population. It did not occur in every colony grouping, but an animal would spend a disproportionate amount of time in one of the inner chambers (Large or Small) and as a result spent little time (less than 10%) in the open-surface chamber (p<0.001) (Figure 39A). The reduced activity of the animal was observed in a qualitative manner; therefore more observations specifically addressing activity and chamber habitation will have to be performed for a more quantitative analysis. The number of times animals moved from one chamber to the other was documented and the identified OMEGA expressed fewer transitions between chambers (data not shown).
The sub-classification was also based on wounding patterns and agonistic interactions. SUB and OMEGA received more wounds on their upper body as well as lower body than DOM (p<0.001) and OMEGA received more total wounds on their body than SUB (p<0.001) (Table 6). Despite these differences, within a given colony the number and severity of wounding was variable. Therefore the data were also analyzed on a per colony basis by calculating the average wounds received during VBS housing by an individual animal as a percentage of the all wounds received in that colony (all 4 males). This analysis revealed that within some colonies animals would receive a disproportionate amount of wounds compared to other colony members and across colonies (p<0.001) (Figure 39B). Furthermore, these identified animals displayed more defensive behavior (p<0.03) (Figure 39C) perhaps due to the fact that they were attempting to defend the territory of the chamber where they resided from those entering the space.

Not all colonies produced an OMEGA; however, some colonies produced more than one based on the above-listed criteria. Although no differences appeared between SUB and OMEGA in percent of time spent in SFC chamber or percent of total wounds (Figure 39 A&B), it is important to note that the determination of OMEGA was on a colony-by-colony basis and behavior of the animals within the VBS (data not shown).
Figure 39. Behaviors associated with the OMEGA phenotype. A) SUB and OMEGA spend less time in the SFC chamber than DOM. B) SUB and OMEGA received more wounds than DOM. C) SUB and OMEGA displayed fewer offensive behaviors than DOM, however OMEGA exhibited more defensive behaviors. $ p<0.05$ vs. DOM.
**Table 6.** Wounds received in the VBS. SUB and OMEGA received more wounds on their upper and lower back than DOM. OMEGA endured twice as many wounds to the upper back than SUB, 50% more on the lower body (although not significant) resulting in a greater amount of wounds to the whole body. See text for p-values. $ vs. DOM, # vs. SUB.
Body weight and composition

DOM, SUB and OMEGA lost a significant amount of body weight compared to CON during VBS housing. Although SUB lost more weight than DOM, OMEGA lost significantly more body than all other groups (p<0.001). The body weight of each group remained constant after 7 days of VBS housing. Upon recovery all groups gained weight. DOM regained weight to CON levels during the 1st week of recovery, SUB recovered body weight to DOM levels at this same rate, but never reached that of CON. After 2 weeks of recovery OMEGA recovered their body weight to SUB levels, but like SUB neither group reached CON body weight (P<0.001). (Figure 40)

Similar to previous reports, all groups lost a significant amount of adipose tissue during VBS exposure compared to CON (p<0.001). This effect was most apparent in OMEGA. Following 1 week of recovery, OMEGA began to gain significantly more adipose tissue and by week 3, both SUB and OMEGA regained more weight as adipose tissue than CON, and OMEGA gained more than DOM (p<0.02, p<0.001 respectively). (Figure 41A).

The most striking characteristic of the OMEGA phenotype is the dramatic loss of lean tissue during VBS housing compared to all other groups (p<0.001). Upon initial recovery OMEGA recover more lean tissue and both SUB and OMEGA have increased lean tissue gain compared to CON by 3 weeks recovery (p<0.001, p<0.01 respectively). (Figure 41B)
Figure 40. % body weight change. All groups lose body weight compared to CON during VBS housing, however this effect is most drastic in OMEGA. DOM recover to CON levels after 1 week of recovery but SUB and OMEGA never reach a similar weight of CON. P<0.001 * vs. CON, $ vs. DOM, # vs. SUB.
Figure 41. Change in adipose tissue. All groups lose a significant amount of adipose tissue during VBS housing, an effect most apparent in OMEGA. OMEGA regain adipose tissue following 1 week of recovery and by the end of recovery both SUB and OMEGA gain significantly more weight as adipose tissue than CON. $P<0.02 \,*\, \text{vs.}\, \text{CON},$ $\dollar\,\text{vs.}\, \text{DOM},$ $\#\,\text{vs.}\, \text{SUB}.$
Figure 41. Change in lean tissue. OMEGA lose significantly more lean tissue than all other groups during VBS housing. This loss is regained during recovery. P<0.01 * vs.

CON, $ vs. DOM, # vs. SUB.
Basal plasma corticosterone and novel restraint stress test

Immediately following VBS exposure animals were challenged with a novel 1-hour restraint stress test. All groups mounted an appropriate stress response except for OMEGA who was hyporesponsive ($p<0.01$) (Figure 42A). Furthermore, OMEGA had increased basal levels of CORT ($p<0.001$) (Figure 42B). At the end of the recovery period, basal plasma levels were similar among groups, and there were no differences found in a novel 1-hour restraint stress challenge (Figure 42 C&D).
Figure 42. Plasma corticosterone. A) All groups responded and recovered to a 1-hour novel restraint stress challenge immediately following VBS housing except for OMEGA who is hyporesponsive. B) OMEGA have increased basal plasma CORT following VBS exposure. C) No differences were found among groups during a 1-hour restraint challenging at the end of recovery. D) Basal plasma corticosterone levels were similar among groups after a 3-week recovery period. P<0.01 * vs. CON, $ vs. DOM, # vs. SUB.
**Food intake**

During the hierarchy formation period (days 1-6 in VBS, see Methods from chapter 3) all groups were hypophagic compared to CON (p<0.001). OMEGA consumed significantly less calories than all other groups. DOM and SUB increased their food intake during the hierarchy maintenance phase (VBS days 7-14); however, OMEGA continued to consume less throughout VBS housing (p<0.001). (Figure 43 A&B)

All groups were hyperphagic upon the initial recovery phase, but only the increased food intake of OMEGA reached statistical significance (p<0.03). By week 3 only DOM remained hyperphagic compared to CON (p<0.01). (Figure 43 A&B)

Compared to the habituation period, CON consumed similar calories, DOM consumed less during hierarchy formation (p<0.01), but more during week 1 of recovery (p<0.05). SUB and OMEGA were hypophagic throughout VBS exposure compared to the habituation period (SUB: p<0.01, OMEGA: p<0.001). Only OMEGA consumed more food during week 1 (p<0.01), but both SUB and OMEGA were hyperphagic compared to habituation during recovery weeks 2 and 3 (SUB: p<0.05, OMEGA: p<0.03).
Figure 43. Food intake. H refers to the average food intake during the 1-week habituation period. All statistical significance identified on the graph compared the OMEGA group to the other groups. This will continue throughout this study on graphs of a similar nature. A) OMEGA consumed less food during VBS exposure compared to all other groups. During the 1st week of recovery, OMEGA were hyperphagic compared to CON. See text for p-values. *vs. CON, $ vs. DOM, # vs. SUB.
**Figure 43B.** All groups consume fewer calories during hierarchy formation, but only SUB and OMEGA remain hypophagic throughout hierarchy maintenance. All groups are hyperphagic during week 1 of recovery, but only OMEGA reached statistical significance. DOM consumed more calories than CON during the final week of the recovery period. See text for p-values.

* vs. CON, § vs. DOM, # vs. SUB.
Meal Patterns

All groups consumed fewer meals during hierarchy formation compared to CON; SUB and OMEGA took fewer meals than DOM (p<0.001). During hierarchy maintenance, OMEGA had a reduced meal frequency compared to CON and DOM (p<0.01). (Figure 44 A&B)

During recovery all groups consumed a similar number of meals. Furthermore, all groups consumed meals at the same frequency compared to habituation during recovery. In contrast, SUB and OMEGA had a reduced meal frequency during hierarchy formation compared to habituation (p<0.001), and OMEGA continued to take fewer meals compared to habituation during hierarchy maintenance (p<0.01). (Figure 44 A&B)

Meal size was initially reduced in SUB and OMEGA during VBS housing (p<0.001), but meal size was similar to CON in all groups during hierarchy maintenance. SUB and OMEGA took larger meals during week 1 of recovery (p<0.01) and SUB continued to consume larger meals during week 3 (p<0.05). (Figure 45 A&B)

DOM meal size did not differ from habituation values during VBS housing, however it was increased throughout the recovery period (p<0.05). SUB and OMEGA had decreased meal size during the hierarchy formation phase compared to habituation (p<0.01) and an increased meal size during recovery compared to habituation (p<0.05),
although during week 2 this trend did not reach statistical significance (p<0.055).

(Figure 45 A&B)

Meal duration was comparable among groups during VBS housing and recovery except during week 1 when OMEGA ate longer meals than CON and DOM (p<0.01). SUB and OMEGA had a longer Inter-MI during hierarchy formation than CON (p<0.01) and OMEGA during hierarchy maintenance than CON and DOM (p<0.001). Throughout recovery Inter-MI was similar among groups except during the final week when SUB and OMEGA expressed a longer Inter-MI compared to DOM (p<0.01). (data not shown)
Figure 44. Meal frequency. A) OMEGA consumes fewer meals during VBS exposure than CON and DOM, but a similar number of meals throughout recovery. See text for p-values * vs. CON, $ vs. DOM
**Figure 44B.** Meal frequency is reduced in all groups compared to CON during hierarchy formation, but only OMEGA continued to take fewer meals during hierarchy maintenance. All groups consumed a similar number of meals during recovery. See text for p-values. * vs. CON, $ vs. DOM
Figure 45. Meal size. A) OMEGA consume smaller meals than all other groups during hierarchy formation, however during recovery, OMEGA consume larger meals than CON. See text for p-values. * vs. CON, $ vs. DOM, # vs. SUB.
SUB and OMEGA consume smaller meals during hierarchy formation but larger meals during the 1st week of recovery. See text for p-values. * vs. CON, $ vs. DOM, # vs. SUB.
Meal patterns: Chamber analysis

Consistent with increased time in the SFC chamber, DOM consumed the majority of their meals in this location. In contrast, OMEGA took most of their meals in the small chamber, whereas SUB took their meals in the large and small chambers (p<0.001). DOM and SUB had similar sized meals in all locations as did OMEGA in the small chamber, however meal size was smaller in the large and surface chamber indicating that OMEGA consume the majority of their energy in the small chamber (p<0.01).

(Table 7)
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<tr>
<td><strong>Number</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>5.10 ± 0.68\textsuperscript{sa}</td>
<td>4.18 ± 0.64\textsuperscript{b}</td>
</tr>
<tr>
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<td>3.49 ± 0.57\textsuperscript{b}</td>
<td>1.18 ± 0.31\textsuperscript{#a}</td>
</tr>
<tr>
<td>Surface</td>
<td>6.11 ± 1.22\textsuperscript{b}</td>
<td>1.06 ± 0.27\textsuperscript{sc}</td>
<td>0.36 ± 0.17\textsuperscript{sa}</td>
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<tr>
<td><strong>Size</strong></td>
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<td></td>
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<tr>
<td>Small</td>
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<td>1.04 ± 0.10</td>
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</tr>
<tr>
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<td>1.02 ± 0.08\textsuperscript{s}</td>
<td>0.46 ± 0.13\textsuperscript{sb}</td>
</tr>
</tbody>
</table>

**Table 7.** Meal patterns within the VBS chambers. DOM take most of their meals in the surface chamber. SUB take more meals in both the large and small chambers, where OMEGA take the majority of their meals in the small chamber. Meal size is similar in all chambers for DOM and SUB, however OMEGA take larger meals in the small chamber. See text for p-values. \$ vs. DOM, \# vs. SUB within that chamber; letters that are not similar indicate significant difference between chambers for that status group.
Meal patterns: Light vs. Dark

DOM and OMEGA ate fewer meals during hierarchy formation compared to CON in the light (p<0.01); however, once the hierarchy was established SUB had an increased meal frequency compared to CON and DOM (p<0.001). In the 1st week of recovery, DOM took fewer meals in the light compared to OMEGA (p<0.03), however no groups differed from CON. (Figure 46A)

All groups consumed fewer meals in the dark during hierarchy formation (p<0.001). During hierarchy maintenance SUB and OMEGA continued to take fewer meals in the dark, an effect that was most significant in OMEGA (p<0.001). Once removed from the VBS, during recovery, all groups consumed a similar number of meals in the dark compared to CON except during the last week in which SUB and OMEGA consumed fewer meals compared to DOM (p<0.01). (Figure 46B)

During hierarchy formation CON and DOM took fewer meals in the light, and CON meals were smaller (p<0.01). During the dark, all groups took fewer, smaller meals compared to CON (p<0.01). Once the hierarchy was established all groups took more of their meals during the dark, except for OMEGA (p<0.001); however, SUB and OMEGA took larger meals in the light (p<0.01). Despite taking more meals in the dark, SUB and OMEGA continued to have a decreased meal frequency in the dark compared to DOM and CON, and OMEGA continued to take smaller meals (p<0.01). Once in recovery, all groups re-established a circadian pattern of feeding by taking the majority of their meals in the dark (p<0.001), however compared to DOM, SUB and OMEGA
took fewer meals ($p<0.01$). There were no differences in meal size during recovery.

(Table 8)
Figure 46A. Meal frequency during the light phase. OMEGA took fewer meals during hierarchy formation compared to CON. During week 1 of recovery OMEGA had an increased meal frequency compared to DOM. See text for p-values. * vs. CON, $ vs. DOM
Figure 46B. Meal frequency during the dark phase. OMEGA took fewer meals during the VBS housing, but took a similar number of meals compared to other groups during recovery except in the final week were their meal frequency was significantly suppressed compared to DOM. See text for p-values. * vs. CON, $ vs. DOM, # vs. SUB.
### Table 8. Meal frequency and size.

During hierarchy formation CON and DOM take fewer meals in the light. All groups take less frequency, small meals in the dark. During hierarchy maintenance, CON, DOM and SUB take fewer meals during the light. However, SUB and OMEGA take larger meals during this time. In the dark, SUB and OMEGA take fewer meals, and OMEGA are smaller. Throughout recovery, all groups take more meals during the dark, however SUB and OMEGA take meals less frequently than DOM. See text for p-values. § vs. dark of same status, * vs. CON, $ vs. DOM, # vs. SUB.

<table>
<thead>
<tr>
<th>Number</th>
<th>Hierarchy Formation</th>
<th>Hierarchy Maintenance</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light</td>
<td>Dark</td>
<td>Light</td>
</tr>
<tr>
<td>CON</td>
<td>2.90 ± 0.39§</td>
<td>11.3 ± 0.68</td>
<td>3.06 ± 0.44§</td>
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<tr>
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<td>5.06 ± 0.90*</td>
<td>2.62 ± 0.41§</td>
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<tr>
<td>SUB</td>
<td>1.86 ± 0.13</td>
<td>2.48 ± 0.42**§</td>
<td>5.34 ± 0.35§</td>
</tr>
<tr>
<td>OMEGA</td>
<td>1.50 ± 0.34</td>
<td>2.02 ± 0.49**§</td>
<td>4.13 ± 0.63</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Size</th>
<th>Hierarchy Formation</th>
<th>Hierarchy Maintenance</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light</td>
<td>Dark</td>
<td>Light</td>
</tr>
<tr>
<td>CON</td>
<td>1.02 ± 0.09§</td>
<td>1.74 ± 0.19</td>
<td>1.07 ± 0.08</td>
</tr>
<tr>
<td>DOM</td>
<td>1.35 ± 0.30</td>
<td>0.99 ± 0.13*</td>
<td>1.19 ± 0.11</td>
</tr>
<tr>
<td>SUB</td>
<td>1.01 ± 0.16</td>
<td>0.69 ± 0.12*</td>
<td>1.34 ± 0.09§</td>
</tr>
<tr>
<td>OMEGA</td>
<td>0.68 ± 0.19</td>
<td>0.40 ± 0.14*</td>
<td>1.46 ± 0.13§</td>
</tr>
</tbody>
</table>
Forced swim test of depressive-like behavior.

Immediately following VBS exposure, DOM and SUB animals spent more time displaying active behaviors in the forced swim test (FST) \( (p<0.05) \) (Figure 47A). After 3-weeks recovery, DOM and SUB continued to be more active in the FST \( (p<0.05) \), but OMEGA displayed less active behaviors compared to DOM and SUB \( (p<0.01) \) (Figure 47B).
Figure 47. Forced swim test. A) Following VBS exposure, DOM and SUB display more active behaviors. B) At the end of the recovery period, DOM and SUB continue to exhibit more active behaviors, however OMEGA demonstrate fewer active behaviors than DOM and SUB. See text for p-values. * vs. CON, § vs. DOM, # vs. SUB.
Hypothalamic and amygdalar NPY expression

All groups had increased NPY expression in the arcuate nucleus following VBS housing (p<0.001); however, NPY expression was similar among groups by the end of recovery (Figure 48A). No differences were found in the NPY mRNA in the dorsomedial hypothalamus at either time-point (Figure 48B).

Directly after VBS exposure OMEGA had increased NPY mRNA expression in the basolateral (p<0.01) and central (p<0.05) nuclei of the amygdala, but not in the medial nucleus. All groups had similar NPY mRNA expression following recovery in each amygdalar nuclei. (Figure 50 A-C)
Figure 48. Hypothalamic NPY mRNA expression. A) All groups expressed significantly more NPY mRNA than CON after VBS exposure, but not following recovery. B) No differences were found in the dorsomedial hypothalamus at either time point. See text for p-values. * vs. CON.
**Figure 49.** Representative photomicrographs of hypothalamic NPY mRNA expression.
Figure 50. Amygdalar NPY mRNA expression. A) OMEGA had increased expression of NPY in the basolateral nucleus compared to CON and SUB after VBS housing, but not after recovery. B) OMEGA expressed significantly more NPY mRNA than SUB in the central nucleus following VBS exposure, but not 3 weeks recovery. C) No differences were found at either time point in the medial nucleus. See text for p-values. * vs. CON, # vs. SUB.
Figure 51. Representative photomicrograph and corresponding coronal brain section of amygdalar nuclei.
Discussion

This study subdivided the SUB population to explore the development of the OMEGA phenotype during VBS housing. Based on behavioral evaluation on a colony-by-colony basis, it was determined that the OMEGA phenotype has distinct physiological, endocrine, ingestive behavior and neurochemical effects of VBS exposure, which potentially indicates that these animals are more vulnerable to stress-related consequences.

Behavior of the OMEGA phenotype.

Initially it was hypothesized that increased time in the open arms of the elevated plus maze would predict hierarchy status of DOM or OMEGA. Despite biasing the colonies such that one of the four males had spent time in the open arms while the others did not, the hypothesis was not confirmed. Although the selected males became DOM or OMEGA, some also became SUB indicating that time on the elevated plus maze prior to VBS housing is not a valid predictor of hierarchy status.

Despite the lack of ability of the elevated plus maze to predict hierarchy status, behavioral analysis during VBS housing confirmed a phenotypic difference between SUB and OMEGA. OMEGA endured a disproportional amount of wounds located on the body (Table 6, Figure 39B) suggesting more defensive behavior. In fact, OMEGA displayed more defensive behaviors in the burrow (Figure 39C). As mentioned above, OMEGA spent a majority of their time in one of the inner chambers (data not shown). This is supported by the fact that the majority of meals and calories consumed by
OMEGA occurred in the small chamber, and very little food was ingested in any other location (Table 7). Together this suggests that OMEGA males may have been defending the small chamber, similar to DOM and the surface area, but that they endured greater consequences such as significant loss of body weight, adipose tissue and lean tissue.

The differential behaviors expressed by DOM, SUB and OMEGA may be a result of dissimilar coping strategies. Coping can be seen as a behavior which allows an animal to deal with a situation or threat while maintaining control (Benus, Bohus et al. 1991). There are two alternative ways of coping: active and passive. Active coping normally occurs in the aggressive animal who will develop routines to handle the environment, will determine the social situation, will flee if defeated, and will actively avoid an aversive situation; all of which leads to the ultimate goal of removing the source of stress, or the stress itself. Conversely, passive coping occurs more commonly in non-aggressive animals that react to the environment, tolerate social interaction, express immobility and withdraw behaviors to reach the ultimate goal of reducing the emotional impact of the situation. It is postulated that actively coping animals have an advantage in a predictable environment, but that active coping can be maladaptive in a changing environment where passively coping animals would succeed (Benus, Bohus et al. 1991). For example, in the case of a predator-prey interaction, a passive coping prey animal may freeze and hide from a predator, whereas an actively coping prey animal may try to flee the scene potentially exposing him for capture. In the current study it is unclear what types of coping strategies the animals express. DOM
are aggressive, determine the social situation and, by defeating intruders, DOM remove
a source of stress from their environment. SUB are non-aggressive and adapt to the
VBS environment by reducing the interactions with DOM by, for example taking the
majority of their meals in the inner chambers. OMEGA are not aggressive and withdraw
from social interactions, yet they actively avoid an aversive situation by remaining in one
of the inner chambers the majority of the time and away from the DOM. Furthermore,
OMEGA do not adapt their behavior by fleeing when they are defeated, as seen by the
increase of defensive wounds. Furthermore, OMEGA spent equal amounts of time
displaying active and passive behaviors in the forced swim test following VBS exposure.
Together, this suggests that OMEGA do not have a successful coping strategy during
VBS housing and this may contribute to some of the endocrine and metabolic
consequences seen in these animals.

SUB from the VBS have previously been sub-classified as non-responders based
on a suppressed glucocorticoid response to a 1-hour restraint test (Blanchard, Sakai et
al. 1993; Albeck, McKittrick et al. 1997; Lucas, Celen et al. 2004). This suggests, as do
the data from the current study, that the dominance hierarchy formed in the VBS may
not always have just a dominant and subordinate classification. Other social species
such as pigs (Hessing, Scheepens et al. 1994), wild rabbits (von Holst, Hutzelmeyer et
al. 1999), cynomolgus monkeys (Macaca fascicularis) (Czoty, Gould et al. 2009;
Riddick, Czoty et al. 2009), squirrel monkeys (Saimiri sciureus) (McKenzie-Quirk and
Miczek 2008), rhesus monkeys (Macaca mulatta) (Wilson, Fisher et al. 2008),
assamese macaques (Macaca assamensis) (Ostner, Heistermann et al. 2008),
chimpanzees (Anestis, Bribiescas et al. 2006) and rats (Pohorecky, Baumann et al. 2004; Pohorecky 2006) form dominance hierarchies but have a rank order, such that there is a dominant and subordinate, but also levels of intermediates between which are dominant to some members of the group and subordinate to others. Of these examples, many indicate changes within the rank in a step-wise fashion, for instance, rabbits have graded decreases in offensive behavior, sexual behavior, and body weight, with each lower ranking animal. On the other hand, all subordinate rabbits have increased CORT compared to the top DOM (von Holst, Hutzelmeyer et al. 1999). This suggests that some characteristics vary by rank whereas others are based on clear dominant and subordinate status. This analysis is also apparent in the current study such that OMEGA have increased defensive behaviors, but OMEGA and SUB have decreased aggressive interactions and both lose body weight and adipose tissue, although the effects are more apparent in the OMEGA population. Therefore, it is important to note that although OMEGA display a more severe SUB-like phenotype, SUB still exist within a VBS.

OMEGA are similar to the previously described non-responders, as OMEGA are also hyporesponsive to a novel 1-hour restraint test immediately following VBS housing. Furthermore, it has been suggested that non-responders are the most severely stressed as they lose the most body weight, have the lowest plasma level of testosterone and greatest extent of adrenal hypertrophy (McKittrick, Blanchard et al. 1994). Similarly, OMEGA have the greatest degree of body weight loss. A dysregulated HPA response is observed in many psychiatric disorders such as major depression and post-traumatic
stress disorder (PTSD) (Yehuda, Giller et al. 1991; Strohle and Holsboer 2003). A diagnostic characteristic of PTSD is enhanced negative feedback observed in animal models of PTSD or following dexamethasone (Dex) administration (Cohen, Zohar et al. 2006; Harvey, Brand et al. 2006; Yehuda, Yang et al. 2006; Kohda, Harada et al. 2007). However, the suppressed HPA response may be more of a symptom of PTSD which endures over time. War deployment, independent of PTSD diagnosis, is associated with enhanced negative feedback, where older trauma survivors of the Holocaust and war veterans diagnosed with PTSD display suppressed CORT following Dex administration, unlike their non-PTSD survivors (Yehuda, Halligan et al. 2002; Golier, Legge et al. 2006; Golier, Schmeidler et al. 2006; de Kloet, Vetmetten et al. 2007).

PTSD is an anxiety disorder and, as previously discussed, anxiety is mediated by the amygdala. fMRI studies indicate that the amygdala is hyperactive in an anxious state and in clinically anxious individuals such as PTSD patients (Etkin and Wager 2007; Garner, Mohler et al. 2009). Two neuropeptides, CRH and NPY both expressed in the amygdala, act in opposition to each other. Intraventricular and intra-amygdalar administration of CRH decrease general activity, social interaction time, time spent on the open arms of the elevated plus maze and increases startle response, all measures of increased anxiety (Dunn and File 1987; Baldwin, Rassnick et al. 1991; Heilig, Koob et al. 1994). CRH antagonists and NPY administration reverse these findings producing anxiolytic behaviors (Sajdyk, Vandergriff et al. 1999; Gutman, Yang et al. 2008; Sajdyk, Johnson et al. 2008). Based on this, and the preliminary elevated plus maze finding which suggested that DOM and OMEGA may exhibit anxiolytic-like behavior prior to
VBS housing, it was hypothesized that these groups of animals would have increased amygdalar NPY expression and promote dominance. In the case of the OMEGA, it was thought that the promotion of dominance from the anxiolytic-like properties of amygdalar NPY would encourage this animal to continually strive for dominance, despite never gaining that status which would result in the increased wounding and loss of body weight. As discussed, it was determined that pretesting with the elevated plus maze does not hold predictive value on VBS hierarchy status. Furthermore, only OMEGA, and not DOM, have increased NPY mRNA expression in BLA and CeA of the amygdala following VBS exposure.

The increase in OMEGA amygdalar NPY expression suggests they would have anxiolytic behaviors. Within the VBS this could be seen as increased social interaction time. However, this was not observed, and in fact, OMEGA seemed to withdraw from social interaction by residing in one of the inner chambers for the majority of the 2-week VBS period. Another way to evaluate anxiety is through the measure of punished responding. The Vogel and Geller-Seifter punished responding paradigms deprive animals of water or food then expose them to a shock chamber with a lever press either immediately following deprivation, or with training. During testing each level press, which provides the previously deprived element, is paired with a footshock (Heilig, Koob et al. 1994; Kask, Harro et al. 2002; Garner, Mohler et al. 2009). An anxiolytic agent will increase the number of punished-responses for water or food, meaning the animal accepts more punishment. Although this cannot be mimicked in the VBS, it is possible that when intruders come into the inner chamber in which OMEGA inhabits, likely in an
aggressive manner, the OMEGA accepts more punishment and does not flee or alter his behavior.

There is also the possibility that interactions and behaviors occurred that were not scored during the video evaluation. Videos were observed for the first 15 minutes of the first 6 hours of the dark. As indicated by meal pattern analysis, the circadian rhythm of both SUB and OMEGA may be altered as they consume larger meals in the light, and take meals throughout the 24-hour light cycle. Therefore, it is possible that critical behavioral interactions took place during the light, a time in which animals are usually less active (Bare and Cicala 1960; Siegel 1961). Ultimately more studies need to be performed to determine the function of amygdalar NPY and CRH in this model of chronic stress. Previously classified non-responders and SUB have increased CRH in the central amygdala (Albeck, McKittrick et al. 1997), this suggests that OMEGA may also have altered CRH expression. Studies designed to explore these neuropeptide changes will reveal what potential role they have in the OMEGA phenotype.

Taken together, it is clear that the OMEGA exists as its own phenotype within the VBS hierarchy, however the mechanism promoting this behavior is unclear. Similar to PTSD models, OMEGA had a suppressed HPA response; however, OMEGA also had increased basal plasma CORT which is not seen in models of PTSD. It is possible that a sustained increase in circulating glucocorticoids exhausts the HPA axis such that an appropriate response cannot be mounted to an acute restraint challenge. Further studies will have to be done to understand the mechanism behind this finding, however
is has been suggested that hyporesponsivity of the HPA axis may be mediated by glucocorticoid receptors in the pituitary and forebrain GABA circuits (Schmidt, Levine et al. 2005; Dent, Choi et al. 2007). Interestingly, OMEGA recover the dysregulated stress response following 3 weeks of recovery when all animals have similar basal plasma CORT levels and a normal stress response. On the other hand, following recovery OMEGA exhibit less active behavior than DOM and SUB in a forced swim test suggesting that this is one, of likely many, enduring effects of VBS stress. To fully understand the OMEGA phenotype and its role in stress research future studies will have to be preformed to explore the disconnects in the current findings. Some of these include the increased basal glucocorticoid level but suppressed HPA response following VBS housing, the potential unsuccessful coping strategy during VBS housing which may prove maladaptive and the role of the anxiolytic expression of NPY within the amygdala.

**Body weight, composition and meal patterns of the OMEGA phenotype**

OMEGA appear to suffer the most metabolic consequences during VBS stress. Although all groups lose weight, OMEGA lost up to 15% of their original body weight and sustained that level through the 2-weeks of VBS housing. Furthermore, OMEGA suffered the greatest loss of adipose tissue, and even more strikingly, appeared to be the only group that loses lean tissue during VBS exposure.
During this time SUB and OMEGA were hypophagic, but SUB began to consume more calories once the hierarchy was established, where OMEGA maintained suppressed food intake. This decrease in food intake was a result of a reduced meal frequency and meal size, which was most apparent in OMEGA. During hierarchy formation (d1-6 of VBS housing) OMEGA took fewer meals in the light along with DOM and all took smaller meals compared to CON. Once the hierarchy was stable (d 7-14 of VBS housing) SUB had an increased meal frequency in the light compared to CON and DOM but all groups took the majority of their meals in the dark except for OMEGA. This suggests that both SUB and OMEGA have altered circadian feeding patterns.

Upon recovery all groups gained body weight. By the end of the first week, DOM had reached CON levels and SUB had similarly recovered body weight to DOM. Despite this neither SUB nor OMEGA recovered their body weight to CON levels during the 3-week recovery period. However, the body weight that was regained by these two groups was predominantly adipose tissue and by the end of the recovery period SUB and OMEGA gained significantly more adipose tissue than CON.

The pattern of food intake likely contributed to this change in body composition. OMEGA were hyperphagic during the initial week of recovery and remained hyperphagic throughout recovery compared to habituation levels of food intake. The increased food intake was a result of increased meal size as there were no differences in meal frequency among any of the groups throughout recovery. Meal size was also larger in SUB during week 1 and 3 and compared to the habituation period.
Furthermore, OMEGA took more meals in the light during the first week of recovery, but overall the disrupted circadian pattern of feeding was recovered as all groups took the majority of their meals in the dark, however SUB and OMEGA had a slight reduction of meal frequency compared to DOM. It is likely that this pattern of ingestive behavior contributes to the gain of adipose tissue as increases in meal size and decreases in meal frequency have been shown to promote the gain of adiposity (Fabry, Hejda et al. 1966; Drewnowski, Cohen et al. 1984; Plucinski, Bruner et al. 1984; Wheeler, Martin et al. 1990; Chapelot, Marmonier et al. 2006).

Although NPY was differentially expressed in the amygdala, all groups housed in the VBS had increased NPY mRNA in the arcuate nucleus following VBS exposure. NPY is best known for its orexigenic properties in the hypothalamus (Kalra and Kalra 2004) and likely promoted the hyperphagia observed in the recovery period. Arcuate NPY levels were similar among groups following recovery. This was not surprising since at the end of recovery animals were no longer in severe negative energy balance. No differences were found at either time point in the dorsomedial nucleus of the hypothalamus. This was unexpected at the recovery time point since NPY in this hypothalamic region has been implicated in increased meal size (Bi 2007) and both SUB and OMEGA took larger meals throughout recovery.

Together, these results further support the classification of the OMEGA phenotype within the VBS hierarchy. Although SUB and OMEGA share similar metabolic consequences to VBS stress and recovery, OMEGA also exhibit independent
effects. Furthermore, the extreme weight, adipose and lean tissue loss along the increased basal plasma glucocorticoid levels and suppressed HPA response indicate that this group may be more vulnerable to VBS stress.

**Conclusion**

In conclusion, clear differences exist among DOM, SUB and OMEGA animals within the VBS hierarchy. There is still no way to predict what status an animal will become before exposed to social stress however it is important to continue to examine possible factors as they may indicate mechanisms which make an individual more vulnerable to stress-related disease. This study has revealed an intriguing phenotype within the VBS hierarchy, which will promote further study into individual differences, stress-related behavior and stress-related mental disease.
References


Chapter 6

General discussion and future directions.
Stress is experienced on an everyday basis in both the animal and human world. Exposure to stress has effects on food intake, body weight and body composition, and these can lead to adverse metabolic consequences; however, the mechanism(s) responsible for this effect is not fully understood. Given that the recent rise in obesity, overweight and other metabolic disorders is concurrent with the increase in experiences of stress, it is clear that studying the relationships among stress, ingestive behavior and body composition are essential if we are to improve health. Animal models of psychological stress, particularly social stress, are useful in exploring these relationships. The visible burrow system (VBS) is a unique model in that the animals are housed in a naturalistic setting and the social stress is derived from the agonistic interactions that occur within the colony. This allows for minimal investigator interference and continual, chronic stress.

Previous findings in the VBS model provided the framework to the work presented in this dissertation by exploring the metabolic consequences of social stress and recovery. The studies presented in the previous chapters took the first step in understanding the potential mechanism behind the changes in body weight and composition previously observed by exploring the role of meal patterns in individual animals during VBS exposure and recovery. In order to do this, a meal pattern program had to be established. Through collaboration with the biomedical engineering department at the University of Cincinnati, we were able to create, validate and employ this software in the work presented in this dissertation.
The overall objective of this work was to establish a valid program and explore the changes in meal patterns during social stress and recovery to test the hypothesis that alterations in meal patterns contribute to the body weight and composition changes observed during VBS stress and recovery. The studies presented in *Chapters 3 and 4* are the first to examine the microstructure of food intake in real-time during social stress and continuously throughout a longitudinal recovery period in rodents.

During the course of these studies an independent observation was made implicating a predictive value of elevated plus maze behavior prior to VBS exposure on hierarchy status. *Chapter 5* explores this discovery and presents a novel SUB-like phenotype within the VBS hierarchy.

This chapter will summarize the main findings from the studies presented in the previous chapters and discuss future directions based on the outcome of this dissertation.

**Validation of the meal pattern program through the examination of high-fat diet consumption**

*Chapter 2* explores the effect of acute exposure to a high-fat diet on meal patterns, body weight and composition. A rich literature describes the detrimental effects of high-fat diet on body weight and composition and explores the mechanisms
behind these changes. Our results confirm the general observation that consumption of a high-fat diet promotes the gain of weight and adiposity and that meal patterns, specifically an increase in meal size, are a key factor in the rapid gain in adipose tissue even after just one week of high-fat diet exposure. What is also important is that our findings, which agree with the accepted effects of high-fat diet consumption, occurred within one week of high-fat diet exposure. The rapid changes, including increased body weight, adipose tissue and larger meal size, indicate that high-fat diet can induce detrimental changes on physiology and satiety signals in a short time. Furthermore, preliminary results also suggest that these effects may also be influenced by age as older rats are more susceptible to the effects of high-fat diet consumption since the changes to meal patterns, body weight and composition were exacerbated in an older population.

These results clearly document how the controls of food intake can be rapidly altered in the face of increased palatability and high-energy macronutrient availability. The data from Chapter 2 hold an important role in the subsequent chapters by confirming previous findings related to meal patterns upon exposure to a high-fat diet. This led us to certify our meal pattern program and parameters as valid and to move to explore, for the first time, meal patterns in real-time under conditions of chronic social stress.

Meal Patterns during chronic social stress and recovery
Chapters 3 and 4 present the first data, to our knowledge, of real-time food intake and meal patterns during chronic social stress exposure and recovery, respectively. Until now, the ability to measure ingestive behavior at the microstructural level was only possible in singly-housed, home-cage environments. Due to this, many of the models used to study the effects of stress on food intake included intermittent bouts of stress and recovery where the animal is exposed to stress, such as a social defeat paradigm, and then returned to their home cage where food intake was monitored. By using microchipped subjects in the VBS model of chronic social stress, we were able to examine ingestive behavior during stress, not immediately after. This allows for a deeper understanding of body weight and composition changes seen as a result of continual chronic stress exposure.

A hallmark of the 2-week VBS stress and 3-week recovery paradigm is the rapid sustained weight loss in SUB animals along with loss of lean and adipose tissue during VBS housing followed by the recovery of body weight and lean and adipose tissue with an exacerbated gain in adiposity particularly in the visceral region in SUB animals during a 3-week recovery period. Previous studies began to explore the likely changes in food intake throughout the paradigm suggesting that the weight loss during VBS housing could be partly attributed to a decrease in food intake, whereas hyperphagia during recovery allowed for the gain of body weight (Tamashiro 2005). Studies presented in Chapters 3 and 4 explore the hypothesis that the pattern of ingestive behavior of SUB animals plays a role in the changes in body mass and composition associated with VBS stress and recovery.
DOM and SUB animals had a decrease in meal frequency and size during hierarchy formation. However, only meal frequency remained suppressed in SUB animals once the hierarchy was established. This resulted in persistent hypophagia of SUB animals throughout VBS housing period. It is likely that this pattern of ingestion mediates, at least in part, the loss of lean and adipose tissue as SUB, although hypophagic overall did steadily increase their food intake during hierarchy maintenance, yet their body weight remained stable at the level lost during hierarchy formation. Other studies have demonstrated that alterations in meal number and size and, perhaps more pertinent to the current results, a subtle reduction in meal frequency alone, can change body composition (Fabry, Hejda et al. 1966; Fabry and Tepperman 1970; Chapelot, Marmonier et al. 2006).

To clarify the extent to which meal patterns per se might mediate the stress-induced loss of body weight, adipose mass and lean tissue, a unique pair-feeding paradigm would be beneficial. Traditional pair-feeding, where a control group is fed the same caloric content as the experimental group, has been useful to delineate the role of reduced food intake in many paradigms. Nonetheless, animals which are pair-fed once a day or even twice a day exhibit a binge-like pattern of food intake where they consume all, or most of the given calories shortly after presented with the food. It is clear that VBS animals do not feed in one or two large bouts a day. Therefore the results of these types of studies, although a good starting point, are not the best control. Yoke-feeding is another pair-feeding type approach where one control animal is paired
to an individual experimental animal. Each time the experimental animal feeds, the partner is presented with the same amount of food attempting to maintain a similar temporal pattern of food intake. However, there is no way to ensure that the partnered animal will consume all, or any, of this ‘meal’. One creative approach attempted to circumvent this uncertainty by a form of yoke-feeding in which an animal would bar press for food and his paired partner would receive an intragastric infusion of the same caloric load, thus mimicking the volume and temporal pattern of the experimental animal (Cox and Powley 1981). This was an innovative approach to the problem of controlling for meal patterns, however an intragastric infusion precludes any signal or mechanism, which resides along the digestive tract prior to stomach from remaining intact. Taken together there is no ‘perfect’ control for animals housed in the VBS, especially for studies related to food intake and metabolic changes.

Body weight-matched controls have been utilized in previous VBS studies in an attempt to separate the effects of body weight loss and stress exposure on the consequences of VBS housing (Tamashiro, Nguyen et al. 2007). One group of animals was food deprived to match the body weight of SUB, but in order to achieve the decreased body weight these animals had to be food restricted to a much greater extent than the voluntary restriction in SUB. These studies provided useful insight by suggesting that food intake alone does not entirely mediate the alterations in body weight and composition, but again, it is unknown to what extent this type of experimenter manipulation might have influenced signals and mechanisms which control food intake and meal patterns. Mimicking the voluntary behavior of an animal in
an unpredictable environment such as the VBS is nearly impossible. Based on this, future studies should consider what role dissimilar meal patterns among control groups may play between VBS animals and controls to the outcome of their study.

Chapter 4 investigates the meal patterns of animals recovering from VBS exposure. Although both DOM and SUB were hyperphagic throughout the 3-week recovery period, DOM achieved the increased caloric consumption through a subtle increase in meal frequency, whereas SUB had a decreased meal frequency that was especially apparent in the dark phase, but more importantly SUB took larger meals. Consumption of larger meals has been shown not only to increase weight gain and adiposity, but also to promote hyperinsulinemia, hyperlipidemia, hypercholesterolemia, and diminished glucose tolerance (Fabry, Hejda et al. 1966; Fabry and Tepperman 1970; Wheeler, Martin et al. 1990; Chapelot, Marmonier et al. 2006). All of these conditions can be reversed with a reduction in meal size and increase in meal frequency, illustrating the significant role in the pattern of ingestion to overall health.

Potential mechanisms involved in the alteration of meal patterns.

Peripheral and central signals control food intake by modulating meal patterns (Smith 1996; Schwartz, Woods et al. 2000; Smith 2000; Woods and Seeley 2000; Woods and D'Alessio 2008). Another goal of the studies presented in this dissertation was to address the question as to what is mediating the voluntary change in meal patterns throughout the VBS-recovery paradigm. We hypothesized that neuropeptide Y (NPY) would be increased at the conclusion of VBS exposure to drive the initial
hyperphagic behavior in recovery. NPY is the most well known stimulator of food intake, has a close relationship with hypothalamic stress circuitry, and has been implicated in promoting an increase in meal size (Campbell, ffrench-Mullen et al. 2001; Kalra and Kalra 2004; Bi 2007; Dimitrov, DeJoseph et al. 2007; Kakui and Kitamura 2007). NPY expression was elevated in the arcuate nucleus of DOM and SUB following VBS housing, implying that NPY likely contributes to the hyperphagic behavior of DOM and SUB following VBS exposure, but both groups remained hyperphagic throughout the 3-week recovery period despite similar NPY expression levels as CON by the end of the 1st week. Therefore, the role of NPY during the VBS-recovery paradigm is unclear.

During VBS housing, we would predict NPY to be elevated throughout the 2 weeks due to the state of negative energy balance in DOM and especially in SUB. This presents a paradox in that food intake remained suppressed specifically in SUB until they were placed into recovery. Once the hierarchy was established, however, DOM consumed a similar amount of calories as CON. This raises two interesting points. First, if NPY expression is elevated, what mechanism is blocking its message to increase caloric consumption in SUB but not in DOM? And second, unlike in the VBS, some models of chronic stress report increases in body weight and food intake; could this be due to intermittent exposure to stress with small recovery periods between sessions in which animals consume more calories?

To address the first point, as discussed in Chapter 3, corticotropin-releasing hormone (CRH) and NPY participate in a negative feedback loop within the
hypothalamus such that the orexigenic NPY neurons within the arcuate nucleus activate the anorexigenic CRH neurons in the paraventricular nucleus; in turn, CRH activation decreases NPY mRNA in the arcuate (Haas, Borgundvaag et al. 1987; Haas and George 1987; Liposits, Sievers et al. 1988; Bchini-Hooft van Huijsduijnen, Rohner-Jeanrenaud et al. 1993; Heinrichs, Menzaghi et al. 1993; Krysiak, Obuchowicz et al. 1999; Schmidt, Liebl et al. 2008). Therefore, although NPY expression is likely up-regulated in both DOM and SUB throughout VBS housing, it is possible that an increase in CRH expression inhibited the orexigenic role of NPY in SUB. However, CRH mRNA expression has been reported to be increased in both DOM and SUB in the paraventricular nucleus following VBS exposure (Albeck, McKittrick et al. 1997), such that, if DOM and SUB have the same CRH and NPY expression profile, at least within the arcuate and paraventricular nucleus, why do DOM recover their food intake and meal patterns, whereas SUB continue to have suppressed caloric consumption and meal frequency? The answer to this remains unclear; however, other neuropeptides, satiation signals and adiposity hormones may be involved.

Agouti-related peptide (AgRP) is co-expressed with NPY in neurons in the arcuate nucleus and can also stimulate food intake (Broberger, Johansen et al. 1998; Hahn, Breininger et al. 1998). Furthermore, AgRP and NPY may be differentially regulated following stress (Helmreich, Parfitt et al. 2005; Kas, Bruijnzeel et al. 2005). Our preliminary results indicate that AgRP is increased in SUB animals compared to levels in DOM and CON following VBS housing and recovery. However, AgRP stimulates food intake via increased meal size (Tang-Christensen, Vrang et al. 2004;
Ilnytska and Argyropoulos 2008; Santollo and Eckel 2008) and therefore, may play a larger role in SUB during recovery rather than throughout VBS housing.

Satiation signals such as cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-1) are released from the gut following ingestion of nutrients and decrease food intake via a reduction in meal size (Ahima and Antwi 2008; D'Alessio 2008; Ruttimann, Arnold et al. 2009; Williams, Baskin et al. 2009). Furthermore, amylin, which is co-secreted with insulin, modulates food intake via a suppression of meal size (Lutz, Geary et al. 1995). Therefore, it is unlikely that any of these hormones are involved with the reduced food intake of SUB during VBS housing since their meal size was similar to that of DOM and CON. To date the only identified orexigenic gut hormone known to increase food intake is ghrelin, and its action increases meal frequency (Mastorakos and Zapanti 2004; Hameed, Dhillo et al. 2009). Therefore it is possible that SUB may have disruptions in ghrelin signaling which would inhibit meal initiation resulting in decreased meal frequency. If this were the case, we would expect either a decrease in circulating plasma ghrelin levels, or a decrease in ghrelin receptor expression on NPY/AgRP neurons. However, a recent study reported that following stress ghrelin levels are actually increased (Zheng, Dobner et al. 2009), leaving this explanation uncertain.

**During recovery**, SUB animals have an increase in meal size. This suggests an impairment in meal termination and could implicate any of the above-mentioned hormones such as CCK, GLP-1 and amylin. We also measured NPY in the dorsomedial nucleus of the hypothalamus (DMH), a region implicated for its role in
increased meal size (Bi 2007; Yang, Scott et al. 2009). NPY expression in the DMH was not different following VBS stress among groups, nor during recovery, but within the SUB population NPY mRNA was increased following 3-weeks compared to the end of the 1st week. Although it is not likely that this is the sole cause of increased meal size during the later part of recovery, it is possible that NPY in the DMH contributes to the increased meal size exhibited by SUB. In support of this theory it has been suggested that the NPY in this nucleus only plays a role in increased food intake following long-term alterations in energy balance, like that experienced in SUB during VBS stress (Bi 2007). However, it is surprising then that NPY is not increased immediately following VBS exposure.

Meal initiation is more environmentally controlled where meal termination is biologically controlled (Smith 1996; Strubbe and Woods 2004). Therefore, it is likely that the reduced frequency in SUB during VBS housing is a reflection of the environment and perhaps that cost of food procurement is too high, where the increased meal size during recovery is due to impaired termination signals which were altered during times of stress.

It has been suggested that models of chronic social stress, which report increases in body weight and adiposity, could be viewed more as intermittent models of stress and recovery since the increased food intake is typically measured following the stress exposure. This is somewhat parallel to the VBS-recovery paradigm such that as soon as animals are removed from the social setting, they immediately consume more
calories. In fact, we would predict similar results of increased weight gain and adiposity if animals were exposed to the VBS for variable and unpredictable amounts of time and allowed to ‘recover’ individually in their home cage between exposures. This is supported by studies in which mice are housed in sensory contact with one another, but are only given physical interaction once a day. Subordinates in this paradigm gain weight and have increased food intake (Bartolomucci, Pederzani et al. 2004; Bartolomucci, Cabassi et al. 2009).

The role of meal patterns in altered body composition

DOM, SUB and OMEGA each go through significant body compositional changes throughout the VBS-recovery paradigm. SUB and OMEGA lose adipose tissue during VBS housing as well as the DOM; however, DOM maintain lean mass. Upon recovery, SUB and OMEGA gain more weight as adipose tissue than DOM or CON.

Subtly altering meal patterns can have significant effects on adiposity, for example, by reducing meal frequency from 4 to 3 meals a day while maintaining the same caloric load, therefore having an increased meal size, resulted in the gain of adipose mass (Chapelot, Marmonier et al. 2006). Furthermore, in a study of school children where meals were served 3, 5, or 7 times per day, the children who ate less frequently gain more weight and had an increase in skinfold thickness (Fabry, Hejda et al. 1966). Alternatively, an increased meal frequency prevents the gain of adipose tissue, even if total caloric intake exceeds energy requirements (Fabry and Tepperman...
1970). It is likely that the increased meal size and slight reduction in meal frequency exhibited by SUB (and OMEGA) during recovery contributes to their development of adiposity. Along the same line, rats who were allowed to gorge following a period of food restriction, similar to SUB while housed in the VBS, gained more weight and more adipose tissue than those rats who were fed in a more frequent, nibbling-like manner (Wheeler, Martin et al. 1990).

Large meals, along with high levels of circulating glucocorticoids, activate lipoprotein lipase (Smolin, Surh et al. 1986; Frayn, Coppack et al. 1995; Bjorntorp 2001; Nieuwenhuizen and Rutters 2008). Lipoprotein lipase is an enzyme, which is responsible for promoting the lipid accumulation in adipocytes. Although it is unknown what that the activity level of lipoprotein lipase is in SUB or OMEGA animals, it is possible that this enzyme is up-regulated following VBS exposure as a result of their increased meal size, and this factor in combination with the residual circulating CORT provides a mechanism or pathway for the increased gain of adipose mass.

Another potential mechanism permitting the divergent gain of adipose tissue among animals is impaired glucose trafficking. Glucose is the main energy source of an organism which maintains homeostatic levels in circulation by trafficking glucose throughout the body. After meals or an oral glucose load, approximately 1/3 of the glucose is distributed to the liver, 1/3 to skeletal muscle and adipose tissue and 1/3 to non-insulin dependent tissues including the CNS (Cherrington 1999).
The fate of glucose tissue uptake (stored or metabolized) depends on the type of tissue and energy status. In the fasting state most glucose is shunted to the CNS and is provided by hepatic gluconeogenesis and glycogenolysis. During meals, insulin is high and lowers plasma glucose by suppressing hepatic glucose production and increasing glucose uptake into peripheral tissues (predominantly muscle). The balance of glucose allocation throughout an organism is disrupted by stress and redistribution of glucose to tissues occurs until the organism regains homeostasis (Peters, Schweiger et al. 2002; Fehm, Kern et al. 2006). Depending on the severity of the stress, changes in glucose trafficking could be enduring and lead to metabolic consequences. Glucose uptake is under the control of a family of proteins called glucose transporters (GLUTs), which are expressed in a tissue specific manner and are differentially regulated to allow for the precise allocation of glucose from circulation. Peripherally, GLUT4 is primarily expressed in muscle and adipose tissue and is transported to the cell membrane through insulin signaling (Huang and Czech 2007; McCarthy and Elmendorf 2007). Once glucose is taken up by the cells of the various tissues it is either immediately used as fuel for the cell or is stored for future use. Alternatively, insulin-stimulated glucose utilization is elevated 2- to 3-fold in both intra-abdominal (epididymal and retroperitoneal) and peripheral (inguinal) fat depots following food deprivation (Cettour-Rose, Samec et al. 2005). Preliminary results indicate that GLUT4 is downregulated in SUB and DOM up to 4 months following VBS exposure (data not shown). Although this does not indicate changes in GLUT4 expression in adipose tissue, it does demonstrate that the experience of VBS stress has long-lasting effects on glucose transport. Further studies are planned to investigate the potential increased expression of GLUT4 in
adipose tissue following VBS housing in SUB animals to determine if the impaired glucose trafficking may contribute to the gain of adipose tissue.

**Increased vulnerability to stress-related disease in the VBS**

*Chapter 5* explored the hypothesis that innate anxiolytic-behavior predicts status within the VBS hierarchy. This was based on an observation that animals that spent more time exploring the open arms of the elevated plus maze later became DOM in their VBS colony (Davis, Krause et al. 2009). Data in *Chapter 5* did not support this conclusion, but they did lead us to identify a novel OMEGA phenotype, which may represent a model to study the increased vulnerability to stress-related disease.

Although not fully developed as a model, the conduct of OMEGA rats within the VBS clearly indicates behavioral differences within the previously described SUB population. The increased consequences of stress exposure such as the severe loss of body weight, adipose and lean tissue, increased basal corticosterone and hypo-responsive HPA axis activity following an acute restraint challenge indicate that OMEGA may be more vulnerable to VBS stress than other members of the colony. The reasons for this vulnerability are unknown, but increased expression of NPY in the amygdala may play a role. Perhaps analogous to the opposing roles NPY and CRH have in the hypothalamus, these neuropeptides also counter each other’s actions within the amygdala. CRH is anxiogenic, whereas NPY is anxiolytic. Many mental disorders indicate altered expression of each of these factors. It is hypothesized that the increased NPY expression in the basolateral and central nucleus of the amygdala in OMEGA may promote anxiolytic behavior within the VBS that increases one’s
susceptibility to agonistic interactions. For example, across many species subordinate behavior includes fleeing from the dominant. Although observations made in Chapter 5 do not indicate that OMEGA engaged the DOM in the open surface area or fled from the DOM’s presence, it is possible that these interactions occurred during a different part of the light cycle than what was evaluated, or that the DOM entered the inner chamber in which the OMEGA spent the majority of his time and that the OMEGA attempted, unsuccessfully, to defend that space, thus leaving him with a disproportionate number of wounds and an unsuccessful coping strategy. It is thought that NPY is increased following periods of chronic stress as an adaptive mechanism to allow organisms to cope with the stressor; however, in the case of the OMEGA it may be maladaptive (Thorsell, Carlsson et al. 1999; Landgraf and Wigger 2002).

Interestingly, during recovery OMEGA regained body weight in a similar manner as other SUB such that neither group achieved CON levels, and both groups gained more adipose tissue than CON. However the latter was exacerbated in OMEGA. Furthermore, OMEGA and SUB had similar meal patterns throughout recovery, implicating an increased meal size and decreased frequency in the gain of adipose tissue seen in these groups. Despite these similarities, OMEGA did display less active behavior in a forced swim test than DOM and SUB following recovery, suggesting that a long-term effect of the OMEGA phenotype could be depressive-like symptoms.

It is important to note that, although results from Chapter 5 indicate that many of the qualities previously associated with SUB in a VBS hierarchy may be driven by a
smaller number of OMEGA, a SUB phenotype still exists. It has previously been suggested that SUB may use their smaller body weight and size as an adaptive mechanism in the face of a socially stressful environment (Nguyen 2007), and further observations from Chapter 5, such as taking meals in the inner chambers and eating throughout the light cycle, show adaptability to the environment indicating a successful coping strategy which allows them to survive during times of aggressive threats.

Similar to DOM, it is unclear what could predict, if anything, the OMEGA status. Recent studies have suggested a genetic link to anxiety and vulnerability to developing disease based on an inherited haplotype associated with the NPY gene in many patients who suffer from anxiety and anxiety-related illness (Heilig, Zachrisson et al. 2004; Zhou, Zhu et al. 2008). Further studies need to be preformed to elucidate the full meaning of this phenotype, but these original findings provide intriguing results.

**Future Directions**

Many findings from the work presented in this dissertation implicate new avenues to pursue which may be responsible for or associated with the changes related to VBS stress. One of these is a disruption of circadian rhythm indicated by the shift in meal taking behavior into the light cycle by SUB (and OMEGA) during VBS housing, which persists, at least partly, into recovery. This shift in ingestive behavior indicates that sleep patterns are disrupted which can be caused by stress, but can also modify food intake (Elomaa 1985; Suchecki, Antunes et al. 2003; Gronli, Murison et al. 2004; Koban and Swinson 2005). This has important implications as sleep deprivation and restriction
can disrupt metabolism and the normal rhythms of hormone release potentially leading to obesity and diabetes (Copinschi 2005; Bray and Young 2007). Orexins, neuropeptides expressed in the hypothalamus, are important for arousal, but have also been implicated in energy balance and food seeking behavior (Sakurai 2003; Sakurai 2005; Selbach and Haas 2006). It is possible that this system is impaired during VBS exposure leading to the disruption of the circadian rhythm. We would expect a down-regulation of orexin expression, which could partially account for the interruption of circadian feeding cycles.

Conclusions and Implications

Few studies have been performed where meal patterns are manipulated; that is, most investigations explore changes in meal patterns resulting from some treatment or experience. To truly gauge the role of meal patterns in alterations of body weight and composition, more studies will have to utilize techniques that manipulate meal patterns. As discussed above, this is no small task as ingestive behavior is a voluntary process and any type of forced changes of feeding, like intra-gastric gavage or oral catheter or even indirect-forced feeding like pair-feeding or yoke-feeding designs will manipulate the signals, which control meal frequency and size.

The VBS model of social stress examines continual chronic social stress. Humans experience similar continual stressful situations such as job loss, war deployment, a financial crisis, bereavement and so on. Therefore it is important to continue to examine this model to further elucidate the endocrine, metabolic,
neurochemical and behavioral changes associated with this type of stress. However, it may also be valuable to induce intermittent exposure to VBS stress and recovery to mimic more everyday stressors such as job and family stress. Both models are relevant to the study of stress and could provide valuable insight into the mechanisms involved in stress-related disease.

With the rapid rise in obesity-related and stress-related disease it is important to understand the relationship between ingestive behavior and stress. The studies presented in this dissertation present the first examination of meal patterns during social stress exposure and throughout recovery, and they have identified a novel VBS phenotype that may be valuable in understanding the individual differences to stress-vulnerability. Consumption of a high-fat diet and taking larger meals both lead to an increase in adiposity. Modern lifestyle in westernized societies promotes these factors through the increase in available, palatable, more calorically-dense foods, to the influence of our social environment which includes social stress and the facilitation of social eating. Each of these affects meal patterns, often by increased meal size. Studies from this dissertation reveal how the increased meal size, promoted by high-fat diet or social stress leads to increased adiposity and together can lead to many health complications.
References


