DISSERTATION

HORMONAL CONTROLS OF OBESITY IN FEEDING AND FASTING HIBERNATING MAMMALS

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2010

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ABSTRACT

HORMONAL CONTROLS OF OBESITY IN FEEDING AND FASTING HIBERNATING MAMMALS

Mammals that hibernate (hibernators) are intriguing models for the study of controls of food intake and adiposity due to their robust circannual cycle of obesity and anorexia. The pathways controlling these cycles in hibernators have not been fully elucidated. In order to clarify the relationships between various hormones, enzymes and metabolic factors, I examined endogenous and experimentally manipulated levels of several factors in hibernators kept under various physiological conditions, including short-term fasting in summer, long-term fasting in winter, and at low and high body temperature. I compared orexigenic factors (such as the hormone ghrelin and the enzyme AMP-activated protein kinase (AMPK)) with anorexigenic compounds (such as leptin and the enzyme acetyl CoA carboxylase (ACC)) at various times of the year and under experimentally manipulated conditions. Ghrelin is an orexigenic hormone produced by the stomach which increases food intake. Leptin is an anorexigenic hormone produced by white adipose tissue (WAT) which decreases food intake. Both of these hormones impact AMPK, a cellular-energy sensing enzyme that increases food intake and fatty acid oxidation through its inactivation of ACC. I found distinct seasonal profiles of these enzymes and hormones that correlated well with the observed life history characteristics of one species of hibernator, the golden-mantled ground squirrel (GMGS,

Callospermophilus lateralis). In spring and summer, when GMGS are normophagic and lipogenic, the hormone profile of hibernators was much like non-hibernating rodents—AMPK and ghrelin increased with fasting, and injected ghrelin caused an increase in food intake with an associated increase in the active form of AMPK (pAMPK). In autumn, when GMGS are hyperphagic and lipogenic, circulating ghrelin concentrations were higher than at other times of the year, and release of leptin from WAT lagged behind fat mass to allow hyperphagic animals to become obese before hibernation. In winter, when GMGS were aphagic and lipolytic, AMPK and ACC activation were higher in torpid than in euthermic animals, circulating leptin concentrations were once again coupled with fat mass, ghrelin was still circulating in the blood, but at lower concentrations than during normophagic seasons, and circulating ghrelin was higher in euthermic than in torpid GMGS. This was the first published investigation of the hormone ghrelin in a true hibernator, and provides a potential explanation of the dramatic seasonal changes in food intake seen in mammals that hibernate.

ACKNOWLEDGEMENTS

Thanks to all my lab mates over the past four years: Melanie Richter, Litto Suu, Ashley Fenn, Tom Barnett, Jenna Bateman, Cara Ostrom, Cassandra Gearhart, Kendra Burdett, and especially my advisor, Professor Greg Florant, for all your help and support.

Thanks to Nikki Glynn in the lab of Dr. Susan Fried for performing the leptin assays at the University of Maryland School of Medicine, and Cassandra Gearhart, Heather Craven, Ashley Fenn, Vanessa Selwyn, Yvonne Diaz, and Nicole Prentice for help with ghrelin, AMPK, and NEFA assays. Thanks to Denise Pearson of Fox Acres Country Club for allowing us to trap ground squirrels on their property.

This research was supported primarily by an NIH grant (#DK 067017-3) to G.L. Florant, and in part from a CNRU grant (# P30 DK072488) to S.K. Fried and a Sigma Xi grant to J.E. Healy.

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Introduction

Interest in the mechanisms behind weight gain and loss is currently on the rise. Concerns about the increasing incidence of obesity-related physiological disorders are encouraging many scientists to examine the pathways leading to these problems. Obesity is impacted by many factors (behavioral, environmental, genetic, and hormonal). Although the pathways are not fully understood, hormones such as insulin, leptin, and ghrelin are known to play an important role in body weight regulation. These hormones appear to interact with each other, and with other metabolic/neuronal factors (NPY, AGRP, POMC, obestatin, somotostatin, adiponectin, FFAs). All of these factors are worthy of further study; however, for the purpose of a doctoral dissertation, I will be investigating the factors ghrelin, leptin, AMPK and ACC, and their effects in mammals that hibernate, since little is known about how these factors impact food intake and obesity in hibernators.

Common abbreviations: ACC = acetyl-CoA carboxylase; AgRP = agouti-related peptide; AMPK = adenosine monophosphate-activated protein kinase; Arc = arcuate nucleus of hypothalamus; BAT = brown adipose tissue; GH = growth hormone; GHS-R1a = growth-hormone secretagogue receptor 1a; GMGS = golden-mantled ground squirrel; IBA = interbout arousal; NEFA = non-esterified fatty acid; NPY = neuropeptide Y; pACC = phosphorylated ACC; pAMPK = phosphorylated AMPK; POMC = pro-opiomelanocortin; PVN = paraventricular nucleus of hypothalamus; Ta = ambient temperature; Tb = body temperature; VMH = ventromedial hypothalamus; WAT = white adipose tissue

Chapter I: Background

I.A.1. *Hibernation*

Hibernation is described as heterothermy in winter time, during which animals undergo multiple-day torpor bouts interrupted by brief periods of euthermia (~36°C for <24 hrs). Mammals that hibernate (hibernators, such as *Spermophilus lateralis* (now *Callospermophilus lateralis* after Helgen et al., 2009) and *Marmota flaviventris*) have a circannual cycle of food intake comprised of three main stages. The hyperphagic stage takes place from late July to early September; during this time animals almost double their food intake and triple body fat stored in white adipose tissue (WAT), becoming clinically obese with greater than 30% body fat (Jameson & Mead, 1964). Toward the end of September, animals completely cease food intake (become aphagic and anorexic) and metabolize primarily fat from October through mid-March. The normophagic stage occurs from March to July; during this time animals reproduce, begin food intake again, and slowly gain back the mass lost during the aphagic stage.

Animals begin undergoing torpor bouts shortly after entering the aphagic stage (mid October). At the beginning of this stage, hibernators' digestive systems shrink with lack of use (Carey & Sills, 1992). During a torpor bout, an animal drops and regulates its body temperature (Tb) close to ambient temperature (Ta) (usually around 5°C in a laboratory setting). The animal slows all metabolic and cardiovascular processes to near zero and undergoes synaptic dissociation (von der Ohe et al., 2007). While hibernating, animals switch from their summer euthermic state of metabolizing primarily

carbohydrates (glycolysis) to utilizing primarily fats (lipolysis) to meet energy demands (Dark, 2005).

I.A.2 *Experimental animals*

Hibernating mammals are useful in physiological research because of their well-developed orexigenic cycle. These animals can be used to conduct research on obesity, diabetes, and other weight-related ailments. Hibernators are interesting models for studies in obesity because, although they become clinically obese every autumn (with greater than 30% body fat), they show few of the pathologies usually associated with obesity (cardiovascular disease, osteoarthritis, diabetes) (Zhou et al., 2001). They do become insulin resistant and develop some arterial plaques during their autumnal hyperphagic period, but these pathologies disappear by spring (Florant et al., 2004, Florant unpub. data).

The golden-mantled ground squirrel (GMGS, *C. lateralis*) is a diurnal mammal that hibernates and has a robust annual cycle of body mass gain and loss primarily driven by changes in food intake. During the hyperphagic prehibernation stage, GMGS double their food intake and increase body mass nearly 50%, mostly in the form of fat (Dark, 2005). During this autumnal period, animals also decrease metabolic rate and activity, which aids in building fat stores (Kenagy et al., 1989). During the aphagic winter hibernation period (October to March), GMGS cease food intake, and many metabolic processes drop to very low rates. During each torpor bout, animals drop Tb to near Ta for several days at a time, and then rewarm to a normal Tb of 36ºC for a few hours (become euthermic) before returning to low Tb (as low as 0ºC). During these euthermic inter-bout arousals (IBAs), animals do not eat and spend most of the time undergoing

sleep (Heller, 2002). In Colorado, some male GMGS emerge from hibernation in early March to set up and defend territories, but females tend to remain torpid through late April or early May (Florant unpub. data). Animals emerge from burrows to mate before recommencing food intake, and then slowly build up body storage reserves over the summer (normophagia). GMGS have a summer weight of approximately 150 g, but increase to an autumn weight of about 350 g by September (Pengelley, 1967). The mechanisms by which hibernators are able to alter their physiology so dramatically over the course of a year are unclear. Martin and Epperson (2008) propose a two-switch model by which hibernators are able to alter gene expression between summer and winter phenotypes, and further alter gene expression within the winter phenotype, allowing metabolic suppression during torpor and metabolic stimulation during interbout arousals.

I.A.3. *Food intake pathway*

There are many factors involved in initiating and shutting down food intake in mammals, but I will describe how the factors of interest to this study are important in the control of hunger and satiation. When an animal fasts, the lack of stimulation of the vagus nerve in an empty stomach stimulates the sympathetic nervous system to release ghrelin from specific cells (X/A- like cells,

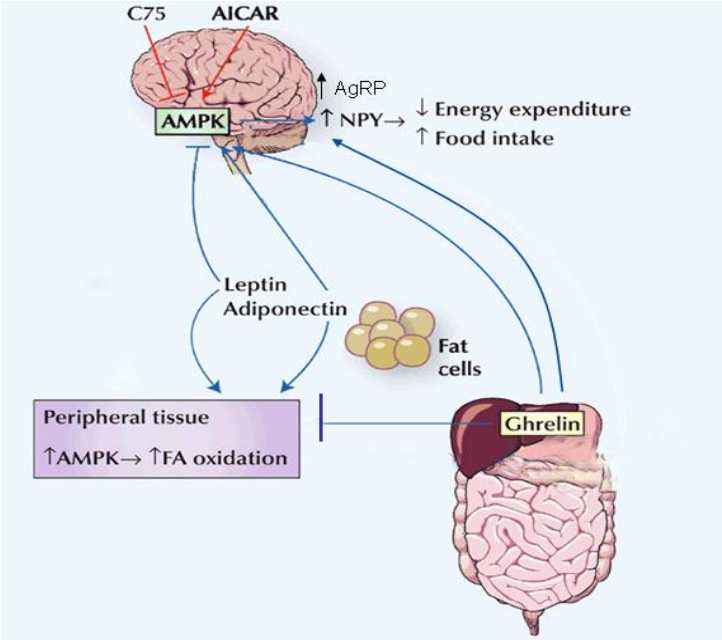

Figure I.1: Food intake pathway diagram (modified from Small et al (2004))

also known as ghrelin cells or Gr cells) in the fundus of the stomach (Dornonville de la Cour et al., 2001). Ghrelin moves from the stomach into the bloodstream, and crosses the blood-brain barrier by an as-yet-unknown mechanism (Fry & Ferguson, 2010). Upon reaching the brain, ghrelin is sequestered in the hypothalamus of the CNS. The arcuate nucleus of the hypothalamus (Arc) contains ghrelin receptors; ghrelin binds to these receptors, which sets off a cascade including the phosphorylation and concomitant activation of AMPK (adenosine monophosphate-activated protein kinase), which in turn stimulates neurons to release the orexigenic NPY (neuropeptide Y) and AgRP (agouti-related protein), and down-regulates the anorexigenic POMC (proopiomelanocortin), resulting in increased food intake and decreased energy expenditure (Minokoshi et al., 2004). Phosphorylation and activation of AMPK also causes the phosphorylation and deactivation of acetyl-CoA carboxylase (ACC). ACC is a committed step in fatty acid synthesis, so its deactivation allows an animal to switch from a lipogenic to a lipolytic state. The afore-described food intake pathway is, in effect, a negative feedback loop, since increased food intake decreases ghrelin levels, possibly by increased stimulation of the vagus nerve.

When an animal fasts, it first metabolizes its readily available carbohydrates (glycogen stored in the liver, stage 1) before utilizing fatty acid oxidation (stage 2); when the fat stores are exhausted, the animal utilizes protein catabolysis (stage 3), breaking down muscle and eventually leading to death. However, hibernators avoid this stage 3 starvation, remaining in stage 2 (lipolysis) for months at a time. In a normal short-term fast (1-5 days), which may occur when environmental conditions prevent an animal from foraging, an animal's blood glucose levels fall, causing an increase in gluconeogenesis

(Cahill, 1976). The pancreas decreases insulin secretion and increases secretion of glucagon, which allows the conversion of liver glycogen into glucose. As the animal uses up its store of glycogen in the liver, it switches to metabolizing lipids. As fat mass decreases, less leptin is secreted into the blood, signaling a low energy state; because leptin inhibits AMPK, lower leptin levels allow greater activation of AMPK. AMPK is activated by phosphorylation, which in turn phosphorylates and inactivates ACC (Carling et al., 1987). Decreased activation of ACC means that less acetyl Co-A is converted to malonyl Co-A, allowing a switch to fatty acid oxidation rather than fatty acid synthesis (for review see Kim & Lee, 2005).

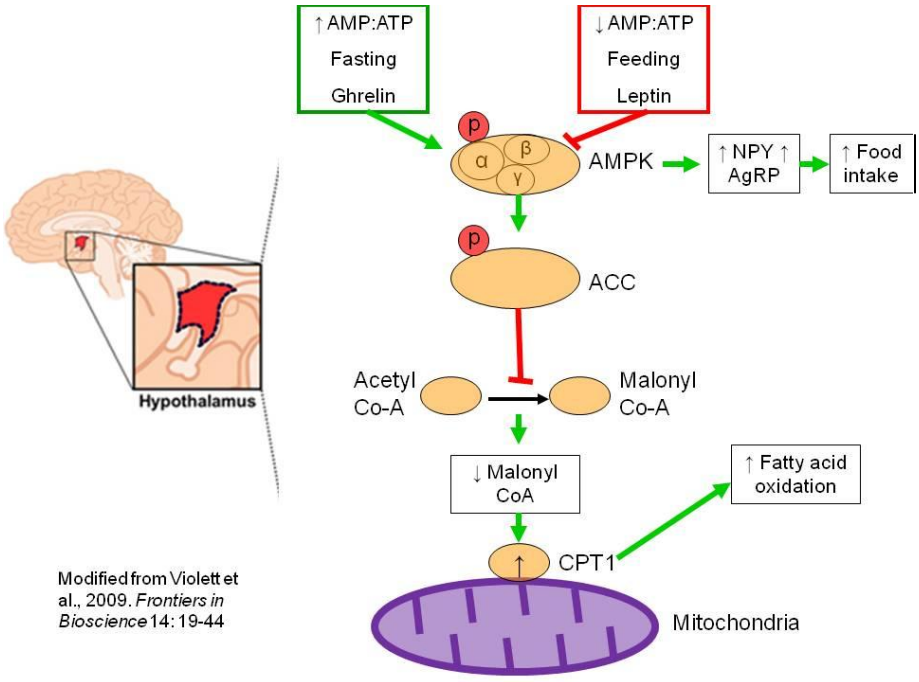

Figure I.2: Diagram of food intake pathway in hypothalamus

I.A.4. *Hypotheses*

My main hypothesis was that the interactions between ghrelin, leptin, AMPK, and ACC help control the food intake and cycle of adiposity in hibernators. I hypothesized that circulating concentrations of leptin would fluctuate with changing white adipose tissue (WAT) stores, but that dissociation of leptin and WAT may occur during the

hyperphagic period of GMGS. I hypothesized that ghrelin would increase with a short term fast, that ghrelin concentrations would be higher during hyperphagic period than in other seasons, that ghrelin injections would cause increased food intake in summer, and that ghrelin injections would not cause a change in food intake during the hibernation season. I further hypothesized that AMPK would increase with short term fasting in hibernators, that injected ghrelin would stimulate hypothalamic AMPK, and that AMPK would be higher in aphagic euthermic hibernators than in torpid animals. Each of these hypotheses is discussed in greater detail in the following sections.

I.B. Leptin

I.B.1. *Physical characteristics of leptin*

The 16 kDa protein hormone leptin was first described in 1994 by Zhang et al. Leptin is named for the Greek word for thin (leptos), since a recessive mutation in the leptin gene (ob) leads to morbid obesity (Zhang et al., 1994). It is produced by adipose tissue (and in smaller amounts by the gastric epithelium and placenta) and generally circulates in levels proportional to lipid stores. This creates a negative feedback loop that alters food intake and may limit the size of the WAT mass in an animal.

I.B.2. *Leptin's role in feeding and fasting*

High circulating leptin levels signal satiety and high fat mass. Leptin down-regulates the orexigenic neuronal factors NPY and AgRP, leading to a decrease in appetite (Schwartz et al., 2000). In most mammals, leptin levels change concurrently with fat mass fluctuation (Florant et al., 2004; Concannon et al., 2001), but Kronfeld-Schor et al. (2000) showed a dissociation between leptin and fat mass in prehibernatory

little brown bats (*Myotis lucifugus*), possibly to allow greater amounts of WAT to be stored and bypassing leptin's satiety effect. In Siberian hamsters (*Phodopus sungorus*), reduced leptin concentrations were required in order for the animal to enter torpor (Freeman et al., 2004). In Arctic ground squirrels (*Urocitellus parryii*), injected leptin was found to reduce pre-hibernation hyperphagia (Boyer et al., 1997; Ormseth et al., 1996). Recent experiments have shown that decreased leptin levels are associated with initiation of food intake in hibernating GMGS (Florant et al. unpublished data).

Leptin resistance (decreased sensitivity to the anorexigenic effects of leptin) occurs in most morbidly obese animals, including humans. This state is often associated with hyperleptinemia.

I.B.3. ***Leptin Receptors***

Leptin binds to cell surface receptor LEPRb in various nuclei of the hypothalamus (especially the ventromedial hypothalamus (VMH)) and acts through the signal transducer and activator of transcription 3 (STAT3) pathway (Ghilardi et al., 1996). Leptin crosses the blood-brain barrier in proportion to its circulating levels, and as such acts as a proximal signal of energy balance in the body. Once leptin is bound to the LEPRb receptor, it activates STAT3, which is phosphorylated and eventually leads to a decrease in appetite. There are six isoforms of the leptin receptor, but only LEPRb has the necessary structure for activation of the STAT3 pathway (Tartaglia, 1997).

I.B.4. ***Leptin's relationship with other energy metabolism hormones***

Leptin generally has an antagonistic relationship with (and opposite physiological effects of) the orexigenic hormone ghrelin. High fat mass creates high leptin concentrations, which seems to decrease circulating ghrelin in some cases, but not all

(Cummings & Foster, 2003). Barazzoni et al. (2003) found that exogenous leptin prevented the typical fasting-induced increase in circulating ghrelin, but other studies have shown that rodents treated with exogenous leptin, creating artificially high circulating leptin concentrations, had a lean body type and increased ghrelin levels (Ariyasu et al., 2002; Toshinai et al., 2001).

Leptin inhibits AMPK in the hypothalamus, specifically in the Arc and paraventricular nucleus (PVN) (Minokoshi et al., 2004), with the effect of decreasing food intake. Peripherally, leptin stimulates AMPK activation in skeletal muscle, with the effect of suppressing ACC and increasing fatty acid oxidation (Minokoshi et al., 2002).

I.B.5. *Hypothesis and experimental design*

Hypothesis 1: Leptin should be positively correlated with WAT stores in hibernators for most of the year, but may dissociate from WAT levels during animals' autumnal hyperphagic season to allow more fat to be stored.

Hypothesis 2: Circulating serum leptin concentrations should decrease with low adipose stores caused by caloric restriction.

In order to test these hypotheses, a group of GMGS was restricted to trap weight by caloric restriction, and another group was allowed *ad libitum* access to food. Body composition of GMGS was measured once per month over a year using total body electrical conductivity (TOBEC). Animals were anesthetized and blood drawn via cardiac puncture at the time of each scan, and serum was removed for analysis by radioimmunoassay (RIA) for leptin.

I.C. Ghrelin

I.C.1. *Physical characteristics of ghrelin*

Ghrelin was first described in 1999 by Kojima et al. as a natural ligand of growth-hormone secretagogue receptor 1a (GHS-R1a). Growth hormone secretagogues are small synthetic molecules which stimulate the release of growth hormone (GH). Ghrelin's ability to stimulate GH secretion led to its name (the root 'ghre' meaning 'to grow') (Kojima et al., 1999).

Ghrelin is a 28-amino acid peptide with an n-octanoylated serine 3 residue (Kojima et al, 1999). It is produced primarily (80%) by specialized epithelial cells lining the fundus of the stomach (Dornonville de la Cour et al., 2001), but is also produced in smaller amounts by the placenta, kidney, pituitary, and hypothalamus (Hosoda et al., 2000; Kojima et al., 1999). It is released into circulation and is involved in various functions, including control of food intake, fat mass, thermoregulation, sleep, and memory (Tschop et al., 2000; Tsubone et al., 2005; Korhonen et al., 2008).

Ghrelin exists in two forms, active (pure-peptide ghrelin) and inactive (des-n-octaonoyl ghrelin (also called des-acyl ghrelin)) (Hosoda et al., 2000; Nishi et al., 2005). While active ghrelin is able to activate GHS-R expressing cells, the non-modified des-acyl ghrelin form is not (Hosoda et al., 2000). In rats, both forms are present in the stomach; in plasma, the active form is the major form (Cowley et al., 2003). In Ay mice (bred to exhibit obesity and diabetes), hyperphagia decreases plasma des-acyl ghrelin (Nonogaki et al., 2006).

I.C.2. *Ghrelin's role in feeding and fasting*

Besides stimulating GH secretion, ghrelin also helps regulate energy balance in rodents and humans. It is known to increase food intake when injected intraperitoneally or intracerebroventricularly in rodents (Andersson et al., 2004; Gluck et al., 2006; Keen-Rhinehart & Bartness, 2005, Kojima et al., 1999; Tschop et al., 2000). In tundra voles (*Microtus oeconomus*), peripherally injected ghrelin increases plasma ghrelin levels and food intake (Mustonen et al., 2002). This increase in food intake is followed by an increase in fat mass in most animals. Peripherally injected ghrelin increases the respiratory quotient in rats, indicating an increased carbohydrate utilization and decreased fat utilization (Nieminen & Mustonen, 2004; Tschop et al., 2000). In adipose tissue, ghrelin antagonizes lipolysis and stimulates the differentiation of preadipocytes, with the effect of stimulating lipogenesis (Choi et al., 2003).

The release of ghrelin occurs in a pulsatile manner, and its circulating levels are dependent on feeding condition (Beck et al., 2002; Toshinai et al., 2001). In rats and humans, plasma ghrelin levels are increased before a meal and decrease immediately following a meal. Anticipation of a meal stimulates the release of ghrelin from the stomach (Drazen et al., 2006).

Fasting results in elevated circulating ghrelin concentrations in mammals including rats, raccoon dogs, and elephant seals have all responded with increased ghrelin levels (Kinzig et al., 2005; Ortiz et al., 2003; Tschop et al., 2000). Siberian hamsters (*Phodopus sungorus*) exhibited a two-fold increase in ghrelin levels during a 48 hour fast, but showed no changes in circulating ghrelin levels during a 6 week period of food restriction (Tups et al., 2004). Many species of mice will undergo shallow torpor bouts

(less than 24 hours, drop Tb a few degrees) in order to conserve energy when subjected to food restriction. Gluck et al. (2006) administered peripheral ghrelin to mice undergoing fasting-induced torpor, with the result of deepening their torpor bouts (extending the time spend torpid and depressing Tb), and through further investigation determined that ghrelin's effects on torpor are controlled by neurons within the Arc of the hypothalamus.

Ghrelin also responds to diet—in rats fed a high-fat diet, plasma ghrelin levels were decreased, while high-carbohydrate diets increased ghrelin levels (Beck et al, 2002; Kinzig et al., 2005). In humans, administration of a high-fat meal led to a prolonged suppression of ghrelin (Otto, 2005).

Plasma ghrelin levels are decreased in obese humans (Drazen et al., 2006), but increased in anorexic individuals (Tolle et al., 2003). Contrarily, Beck et al (2003) showed an increase in ghrelin in obese Zucker rats. Ghrelin serum concentrations were increased in the stomach and hypothalamus of obese rats compared with controls, but the expression level in the stomach was 100 times higher than in the brain (Beck et al, 2003).

I.C.3. *Ghrelin Receptors*

Ghrelin is acylated to its active form by ghrelin o-acyl-transferase (GOAT) (Yang et al., 2008). The acylated form interacts with receptor GHS-R1a, a seven-transmembrane G protein-coupled receptor (Kojima et al., 1999). This receptor is found primarily in the hypothalamus, specifically on NPY/AgRP-expressing neurons in the Arc and VMH (Cowley et al, 2003). These neuropeptides are orexigenic, and their release is stimulated by active ghrelin (Nakazato et al., 2001).

I.C.4. *Ghrelin's relationship with other energy metabolism hormones*

Leptin generally has an inhibitory effect on ghrelin, both on gastric secretion and the effects of ghrelin on food intake (Kalra et al., 2005; Tsubone et al., 2005), but reports of this effect are not universal (see above discussion in section I.B.4). Injected ghrelin in rats activates AMPK in the hypothalamus and heart, but inactivates AMPK in liver and WAT (Andersson et al, 2004; Kim & Lee, 2005; Kola et al., 2005).

Ghrelin levels in humans and rats have been shown to decrease with administration of somatostatin, which appears to be a ghrelin inhibitor (Silva 2005).

Obestatin is a recently discovered peptide that is encoded by the same gene that encodes for ghrelin. This gene produces a protein that breaks into two smaller peptides, ghrelin and obestatin; ghrelin, as described above, has an orexigenic effect, while obestatin's effect is anorexigenic, and similar to that of leptin (Zhang et al., 2005). The fact that these two peptides produced by the same gene have opposing effects on weight regulation has possible interesting ramifications on our research on feeding and fasting in hibernating mammals.

I.C.5. *Hypothesis and experimental design*

Since ghrelin plays such an important role in food intake and energy regulation in other rodents, it is likely to be important in rodent hibernators. The robust and repeating annual cycle of obesity and anorexia exhibited by hibernators provides an opportunity to examine changes in ghrelin in an animal with natural physiologically regulated fluctuations in food intake.

Hypothesis 1: Circulating ghrelin concentrations should fluctuate over the course of a day, decreasing after food intake and increasing between periods of food intake.

Hypothesis 2: Ghrelin should be found in high concentrations in the blood during the autumnal hyperphagic period of hibernators, and then decrease as the animal enters the hibernation period.

Hypothesis 3: During a short-term fast (from 16-48 hours), ghrelin levels should rise; during long term fasting in hibernation, ghrelin concentrations should be lower than in the normophagic euthermic spring and summer seasons.

Hypothesis 4: Peripherally injected ghrelin should increase feeding during normophagic and hyperphagic seasons, but should not have an effect on animals in the aphagic hibernation season.

Hypothesis 5: Injected ghrelin should also impact the behavior of GMGS, causing an increase in locomotor activity in addition to feeding-related behaviors.

In order to test these hypotheses, blood was drawn from GMGS under various conditions: once per month under normal feeding conditions, every two hours over a 24-hour period, after a short-term fast in summer, and from winter torpid and winter euthermic animals. Plasma or serum was removed and enzyme immunoassays (EIA) were used to determine circulating ghrelin concentrations under various conditions. Ghrelin was injected intraperitoneally (IP) into GMGS once per season (spring, summer, autumn, winter), animals' behavior was monitored, and animals were sacrificed for blood

and tissue collection two hours after injections to test changes in circulating ghrelin and in total and phosphorylated AMPK and ACC.

I.D. AMPK and ACC

I.D.1. *Physical characteristics of AMPK & ACC*

AMP-activated protein kinase (AMPK) is a heterotrimeric enzyme that plays a role in cellular energy sensing, and is produced in virtually all tissues, including the liver, brain, skeletal muscle, and adipose tissue. It has three protein subunits (α, β, γ), each of which plays a specific role. The γ subunit is responsible for sensing the cellular AMP:ATP ratio; increased concentrations of AMP (which occur when cellular energy is low) cause a conformational change in the γ subunit, which exposes the active site Thr-172 on the α subunit. AMPK is then phosphorylated and activated, leading to an increase in food intake (Xue & Kahn, 2006).

Acetyl-CoA carboxylase (ACC) is a multi-domain enzyme that catalyzes the conversion of acetyl-CoA to malonyl-CoA, which is an important step in lipid storage. ACC is inactivated by reversible phosphorylation (Carling et al., 1987).

I.D.2. *Roles of AMPK & ACC in feeding and fasting*

AMPK is an important metabolic regulator that senses decreasing cellular energy status and responds by stimulating ATP producing pathways, such as fatty acid oxidation and glycolysis. AMPK stimulates fatty acid oxidation by phosphorylating and inactivating ACC. Inhibition of ACC decreases malonyl-CoA levels; since high malonyl-CoA levels are needed to inhibit carnitine palmitoyl transferase-1 (CPT1) and decrease

mitochondrial oxidation of fatty acids, inhibition of ACC allows increased fatty acid oxidation. The non-phosphorylated, active form of ACC increases fatty acid synthesis.

In peripheral tissues, AMPK regulates a variety of metabolic pathways that support catabolic (ATP-producing) processes, but acts differently in each tissue type, and sometimes by tissue location. Mice fasted for 6-24 hours had increased pAMPK in epididymal WAT but not in subcutaneous WAT deposits (Sponarova et al., 2005). In Wistar rats, short-term fasting (19-39 hours) resulted in an increase of pAMPK and pACC in WAT, but not in liver or muscle tissue (Kajita et al., 2008). Sprague-Dawley rats fasted for two days had increased pAMPK in the VMH of the hypothalamus (Murphy et al., 2009). Long term caloric restriction in Wistar rats appeared to decrease pAMPK in the liver, but had no effect on pACC (To et al., 2007).

Few have studied the effects of AMPK in hibernating mammals. Horman et al. (2005) found that during hibernation (a long-term fast), AMPK was activated in white adipose tissue (WAT) in thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*), but not in liver, muscle, brown adipose tissue (BAT) or brain. However, this experiment examined AMPK in the brain in a very non-specific way, and more information is needed on AMPK in specific areas of the brain, such as hypothalamus, where AMPK acts as a fuel sensor. Recent experiments in our lab (Florant et al., 2010) showed that central infusion of the AMPK agonist 5-aminoimidazole-4-carboxamide 1 B-D-ribofuranoside (AICAR) in winter aphagic marmots caused an initiation of food intake.

I.D.3. *AMPK & ACC reactive neurons*

AMPK interacts with ghrelin and leptin in the Arc and paraventricular nucleus (PVN) of the hypothalamus (Minokoshi et al., 2004). Insulin inhibits AMPK activity in

the lateral hypothalamus, ventromedial hypothalamus (VMH) and dorsomedial hypothalamus (DMH) (Minokoshi et al., 2004).

I.D.4. *AMPK & ACC's relationship with other energy metabolism hormones*

Injection of leptin, which reduces food intake, decreases AMPK activity in the hypothalamus, while administration of ghrelin increases hypothalamic AMPK activity in rats (Andersson et al., 2004). Injection of AICAR (a synthetic AMPK activator) directly into the hypothalamus increases food intake in a time dependant manner (Andersson et al., 2004; Dagon et al., 2005).

AMPK also appears to be affected by food intake. Treatment of diet-restricted mice with leptin reduced AMPK phosphorylation (Dagon et al., 2005). In another experiment with mice, diet induced obesity (DIO) was found to upregulate AMPK in muscle and hypothalamus, and impair effects of leptin on the AMPK signaling pathway (Martin et al., 2006). In rats, dietary polyunsaturated fatty acids (PUFA) were found to enhance activity of AMPK in the liver (Suchankova et al., 2006).

I.D.5. *Hypothesis and experimental design*

AMPK is part of a regulatory pathway controlling feeding and fasting in hibernators; it should decrease in fasting animals compared with fed controls.

Hypothesis 1: AMPK is likely to be down-regulated during the hibernation season when animals are aphagic as compared to the summer when animals are normophagic.

Hypothesis 2: In tissues such as liver, muscle, WAT, and BAT, AMPK is likely to be lower in torpid than in euthermic winter-acclimated animals since protein expression is generally down-regulated when animals are at low tissue temperature.

In order to test these hypotheses, GMGS were submitted to a short term summer fast (either 0, 1, 3, or 5 days) and sacrificed for blood and tissue samples. GMGS were also sacrificed for tissues during the winter aphagic period, both at low tissue temperature (during a torpor bout) and during an interbout arousal (euthermic).

I.E. Statistical analyses

Differences between euthermic and torpid winter animals in ghrelin, AMPK, ACC, fatty acids were tested with Student's t-test. Effects of ghrelin or saline injection on food intake during each season were compared using Student's t-test. Differences between summer fasting states in ghrelin, AMPK, and ACC were compared using a 1-way analysis of variance (ANOVA) with a Student-Neuman-Kuels post test. Differences between ghrelin injection and saline injection in behavior were determined by 1-way ANOVA followed by a Bonferroni post-test. Differences between months for ghrelin, leptin and body composition were determined using a repeated measures ANOVA. All differences between groups were tested using GraphPad Prism 5, and were considered significant at $p \leq 0.05$.

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Chapter II: Leptin and fat mass

II.A. Changes in serum leptin concentrations with fat mass in golden-mantled ground squirrels *(Callospermophilus lateralis)*[1]

II.A.1. *Abstract*

Mammals that hibernate present a unique model for investigating mechanisms of food intake and cycles of body mass. The hormone leptin, usually closely linked to white adipose tissue levels, is thought to be an important feedback signal to the CNS to control food intake and consequently, energy balance. We compared serum leptin and fat mass levels in two groups of golden-mantled ground squirrels (*C. lateralis*) during the pre-hibernation period. One group was provided with *ad libitum* food, while the other group was kept on a restricted diet that prevented body mass gain. We found that serum leptin increased significantly in the control group and there was a significant increase in body fat. However, only a small increase in serum leptin occurred in the restricted-diet group and body fat mass levels remained low. These results suggest that serum leptin concentrations reflect total body fat during this period of the body mass cycle.

[1] Published as: Healy, J.E., Richter, M.M., Suu, L., Fried, S.K., Florant, G.L. Changes in serum leptin concentrations with fat mass in golden-mantled ground squirrels (*Spermophilus lateralis*). In: Lovegrove, B.G. and McKechnie, A.E. (eds.) 2008. *Hypometabolism in animals: torpor, hibernation and cryobiology*. University of KwaZulu-Natal, Pietermaritzburg.

II.A.2. *Introduction*

Golden-mantled ground squirrels (GMGS) are mammalian hibernators that have been used as a model of body mass weight gain and loss for many years (for a review see (Dark, 2005)). Hibernators go through alternating cycles of lipogenesis and lipolysis during which their food intake levels rise and fall correspondingly. It is well known that the body mass of mammalian hibernators peaks in autumn prior to hibernation, with the nadir occurring in late spring once animals have ceased entering torpor. In the adult GMGS, body mass can double from summer to autumn and a significant proportion of that increase is in the form of triacylglycerol (e.g. fat) in white adipose tissue (WAT). Prior to winter, these animals greatly decrease food intake, so the energy demands of undergoing multi-day torpor are met primarily by metabolizing the stored fats in WAT. This body (and fat) mass cycle has been extensively documented as a circannual rhythm (Mrosovsky & Faust, 1985; Dark, 2005). However, WAT is not only important as an energy store for hibernators and other mammals, including humans—it also secretes several hormones, including leptin, adiponectin, and resistin, that can regulate food intake and energy balance. Circulating levels of leptin appear to be proportional to body fat content in non-hibernating rodents and humans (Ahima & Flier, 2000; Badman & Flier, 2007).

Leptin is associated with the annual cycles of food intake and adiposity in seasonal mammals, including mammals that hibernate. As a hormone closely correlated with WAT levels, leptin may provide a crucial feedback signal from WAT stores to the central nervous system (CNS) reflecting total body lipid levels and energy balance (Dark, 2005). A major site of leptin action is the hypothalamic arcuate nucleus (Arc) wherein

leptin interacts with two distinct populations of neurons: those that contain orexigenic peptides neuropeptide Y (NPY) and agouti-related peptide (AgRP), and more lateral neurons that express proopiomelanocortin (POMC). As brain leptin levels increase, this produces a decrease in NPY and AgRP expression, and increases POMC expression, leading to a change in food intake. Leptin and perhaps adiponectin inhibit 5'-AMP-activated protein kinase (AMPK) activity specifically in the ARC and paraventricular nucleus (PVN), and this inhibition is essential for leptin's anorexigenic and weight loss effects (Minokoshi et al., 2002; Minokoshi et al., 2004).

Previous studies performed on hibernators have shown that leptin levels are positively correlated with fat mass, with some exceptions. In woodchucks (*Marmota monax*), serum leptin increases with body mass and peaks while food intake is declining (Concannon et al., 2001). Similarly, in yellow-bellied marmots (*M. flaviventris*), leptin was positively correlated with fat mass and fat cell size, which were highest during a period of hyperphagia in the autumn just prior to hibernation (Florant et al., 2004). However, in prehibernatory little brown bats (*Myotis lucifugus*), leptin secretion is dissociated from fat mass—leptin increases before the increase in adiposity, suggesting a state of leptin resistance during the pre-hibernation hyperphagic period (Kronfeld-Schor et al., 2000). Other studies have shown that exogenous leptin inhibits food intake, both pre- and post-hibernation in ground squirrels (Ormseth et al., 1996; Boyer et al., 1997) and also inhibits torpor in seasonal mammals (Geiser et al., 1998; Freeman et al., 2004).

To determine if leptin levels can be dissociated from fat mass in GMGS, we measured serum leptin levels during the autumnal hyperphagic period of body mass gain in two groups of animals kept under different feeding regimes: ad libitum control and

restricted diet groups. We hypothesized that serum leptin levels would rise with increased adiposity in control GMGS, but animals maintained on a restricted diet would not show the typical autumnal rise in serum leptin levels because they would not have increased their body fat.

II.A.3. *Methods and Materials*

Adult GMGS were trapped in the Red Feather Lakes region of Colorado between May and June of 2006 and maintained in our animal facilities at CSU under an approved animal care protocol. GMGS were fed a rodent diet (Teklad 8650) and randomly assigned to two feeding regimes—one group (control, n=6) was fed ad libitum, while in the other group (constant mass, n=5) each squirrel was kept at trap mass (approximately 200 g as determined on 29 June 2006) by restricting food intake to ~7 g/day starting on July 13. Constant mass GMGS were weighed daily until mass stabilized near trap weight, then were weighed weekly. Until October, animals were kept at 20±3 ºC under a natural photoperiod, after which the temperature was lowered to 5ºC and animals were kept in low red light (<20 lux). From June through March, GMGS fat mass was determined monthly by total body electrical conductivity (TOBEC) using a model SA-2 EM scanner (EM Scan, Springfield, IL) as previously described (Pulawa & Florant, 2000). Average percent body fat was determined by dividing the average fat mass (in grams) by the total body mass. All EM scans were performed in the morning after animals had been fasted overnight to ensure that the stomach was empty of food. Animals were anesthetized (IM) with a cocktail of ketamine, acepromazine and xylazine (75:24.5:0.5% respectively); body mass (grams) was measured and fasted blood samples were collected. Whole blood was centrifuged to obtain serum, which was then stored at -80ºC. Leptin concentrations

(ng/ml) were determined by a multi-species radioimmunoassay (RIA) (Linco Research, cat # XL-85 K). All statistical comparisons were performed using SAS 9.1. Standard *t*-tests were done for pair-wise comparisons, and differences were considered significant at the $p<0.05$ level.

II.A.4. *Results*

GMGS were separated in July into control and constant mass groups. By August, the two groups were significantly different ($p<.05$) in body mass and remained so through November. From August to October, the control squirrels on an *ad lib* diet gained ~80 g (a 45% increase in body mass) while the constant mass squirrels on a restricted diet remained near to the trapped mass (Figure II.A.1).

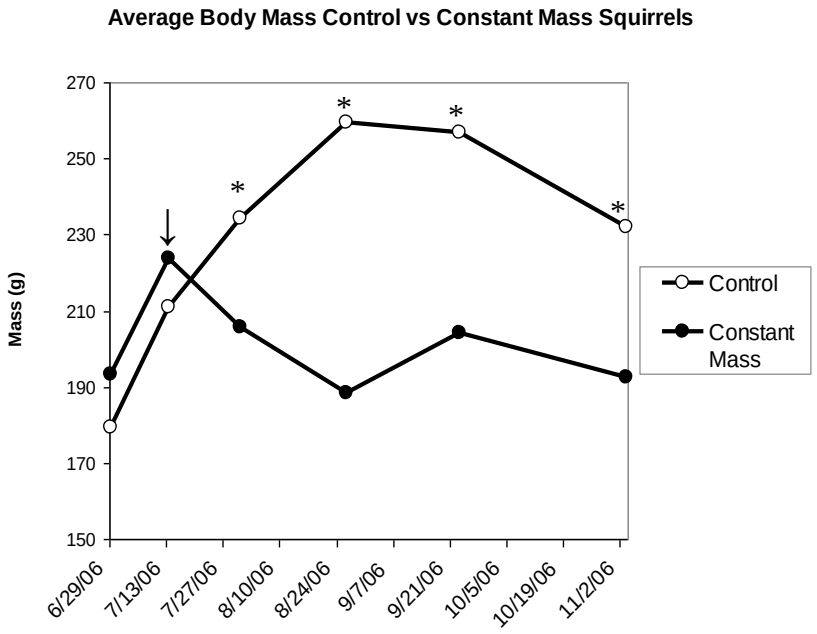

Figure II.A.1: Comparison of body mass of control versus constant mass GMGS from June-November, 2006. ↓= separated groups; *=groups significantly different ($p<0.05$). Control n=6, CM n=5. (From Healy et al., 2008)

Fat mass values increased significantly between July and August for control squirrels, and between control and constant mass squirrels from August to November ($p<0.05$) (Figure II.A.2.a). Both groups of animals had roughly 32% body mass as fat at the beginning of the experiment (July). However, in constant mass GMGS the proportion of fat to body mass declined to 21.9% by November, whereas the control GMGS was

33% fat mass. Serum leptin values are statistically different between September and October for constant mass squirrels and between July and November serum leptin increased four-fold in control squirrels ($p<0.05$) (Figure II.A.2.b). In the constant mass squirrels, leptin levels stayed low June through November, showing a moderate increase in October, but remaining significantly below ($p<.05$) control leptin levels (Figure II.A.2.b). Leptin increased significantly in control animals from July to September. In CM animals, the rise in the mean leptin levels between September and October was not significant.

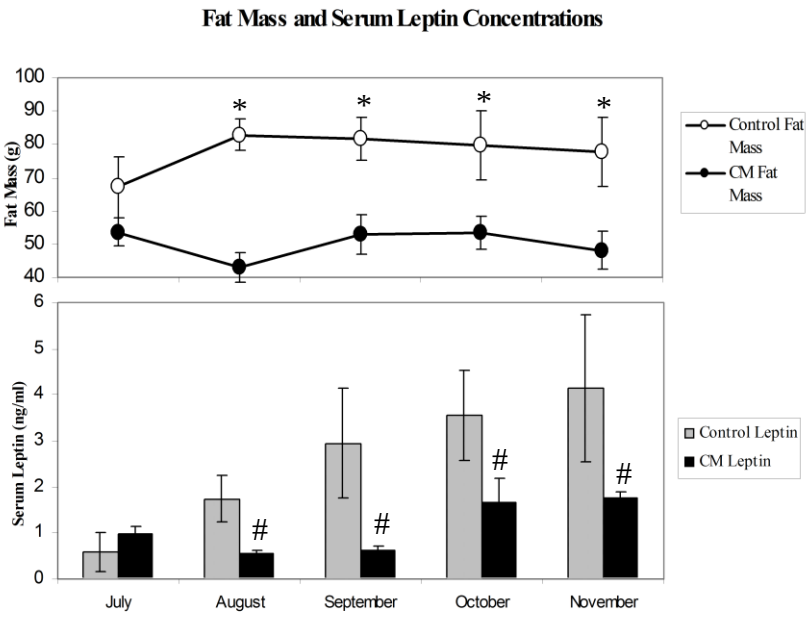

Figure II.A.2: Mean fat mass and leptin values in control and in constant mass GMGS from July-November, 2006; *=control fat mass significantly higher than CM; # = control leptin significantly higher than CM ($p<0.05$). (From Healy et al., 2008)

In control animals, fat mass increased significantly between June and August ($p=0.004$), but leptin concentrations remained low and stable until September, when they increased significantly over summer levels ($p=0.05$). Serum leptin remained high through November and then began to decline in accordance with falling body mass throughout the rest of winter. In February, toward the end of the hibernation season, animals lost lean mass as well as fat mass (Figure II.A.3).

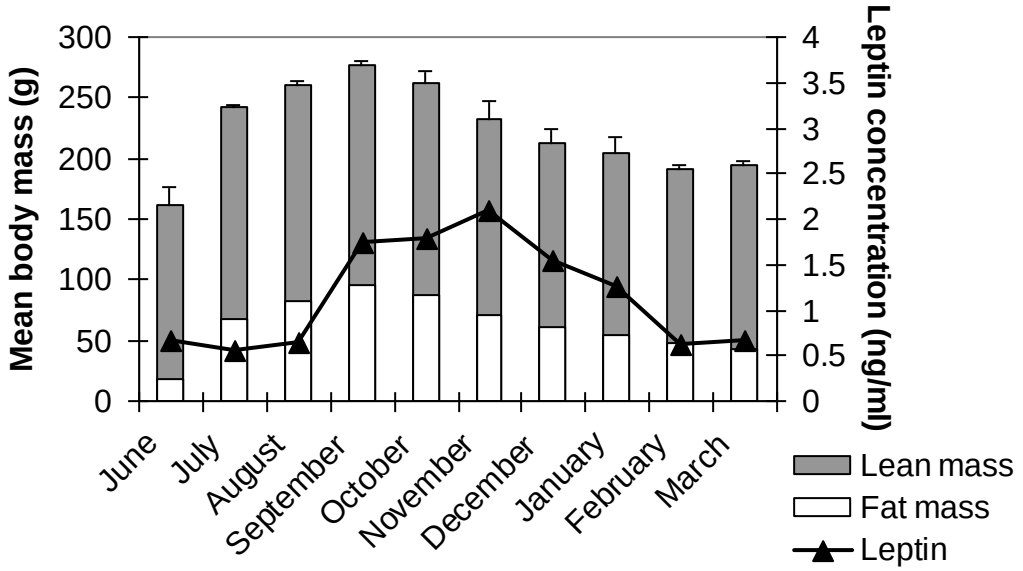

Figure II.A.3: Mean body composition (fat mass and lean mass) and leptin concentrations by month in control GMGS from June-March, 2006-2007.

II.A.5. *Discussion*

During the pre-hibernation period, control GMGS maintained a higher body mass, greater fat mass, and higher leptin levels than the constant mass squirrels maintained on a restricted diet. Interestingly, the percentage of the body mass that was fat (e.g. % body fat) was not significantly different between the control and constant mass squirrels. This result has been observed in GMGS before (for a review, see (Dark, 2005) and was therefore not unexpected. Furthermore, food intake for control animals was greatest prior to the peak in body and fat mass (data not shown) as has been previously reported for GMGS (Dark, 2005). This delayed increase in body mass is assumed to be a combination of reduced physical activity and a reduction in metabolism (Pengelley & Fisher, 1963). Control and constant mass animals appeared to enter torpor at about the

same time, but this observation would have to be confirmed with body temperature measurements.

Total average fat mass levels in control GMGS increased significantly from July to August ($p<0.05$), then remained fairly constant through November. The percentage of body fat in control animals was not significantly different between July and November (31.7% vs. 33.0%). However, in constant mass animals the percent body fat declined between July and November (31.9% vs. 21.9% respectively). Possible explanations for this decrease in percent body fat in constant mass animals might include, but are not limited to, a change in metabolic rate, greater activity levels, or changes in endogenous energy usage. Whether specific WAT fat depots differentially changed in size as seen previously in the literature (Dark et al., 1984; Dark et al., 1989) is unknown.

This is the first report of serum leptin levels in GMGS maintained under different body mass conditions. We found that serum leptin levels were significantly elevated ($p<0.05$) between July and November in control GMGS. In constant mass GMGS, leptin levels increased slightly between September and October. This increase was at the limits of the assay detection and therefore we are cautious about reporting significance. Leptin levels remained significantly lower in the constant mass GMGS compared with control animals from August to November ($p<0.05$). Previous studies in other ground squirrels (i.e. *Spermophilus*) have demonstrated that leptin levels are positively correlated with body mass post-hibernation (Buck & Jenkins, 2005) and that leptin can influence feeding during the pre- and post-hibernation phases (Ormseth et al., 1996; Boyer et al., 1997). During the pre-hibernation period when control GMGS were gaining fat mass, serum leptin levels remained low from June-September, then increased significantly between

August and September, lagging behind the increase in fat mass. This supports previous findings for most mammals that serum leptin levels increase with increasing adiposity (Ahima & Flier, 2000; Rousseau et al., 2003), but more specifically supports the findings of Kronfeld-Schor et al. (2000) that leptin may be dissociated from fat mass stores in prehibernatory animals. This delay in increasing leptin concentrations may allow animals to continue hyperphagia and lipogenesis when normally high fat mass levels would increase leptin and shut off food intake. We believe that serum leptin levels in constant mass GMGS remained low during the prehibernatory period because body fat in these animals remained low throughout the period during which food intake increased. Consequently, this suggests that absolute fat levels must rise in order for serum leptin levels to increase. However, our findings are correlative and other interpretations are possible. For example, NPY acting downstream from leptin could be altered leading to an increase in body fat (Boswell et al., 1993). Future studies of AMPK, other hormones and kinases associated with the food intake pathway and the activity of ARC neurons can provide important information about the control of food intake in hibernating mammals.

In conclusion, GMGS that are restricted in food intake do not deposit a significant amount of absolute body fat and therefore have low serum leptin levels. The consequence of having a low leptin level might be to stimulate food intake and delay the onset of hibernation. In this study, we do not report the exact timing of the initial torpor bout and thus do not know if the initiation of torpor was delayed as shown in previous studies on food-restricted hibernators (Mrosovsky, 1980). The relationship between serum leptin levels and total body fat should be investigated further given the unique animal model that hibernators present for the study of obesity.

II.A.6. *References*

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Chapter III: Levels and effects of ghrelin

III.A. Plasma ghrelin concentrations change with physiological state in a sciurid hibernator *(Callospermophilus lateralis*)[2]

III.A.1. *Abstract*

Ghrelin is a recently discovered hormone which has profound effects on food intake and lipogenesis in mammals. In all mammals studied thus far, plasma ghrelin concentrations are increased before a meal and decrease immediately following a meal; ghrelin levels increase with fasting. The golden-mantled ground squirrel *Callospermophilus lateralis* (formerly known as *Spermophilus lateralis* (see Helgen et al., 2009)) is a diurnal hibernator which has a robust annual cycle of body mass gain and loss that is primarily controlled by food intake. We hypothesized that in spring, summer, and autumn, the endogenous ghrelin concentrations of hibernators would be similar to those of non-hibernators, but that during the winter hibernation season plasma ghrelin concentrations would be low or undetectable. We found that peripherally injected ghrelin significantly increased food intake in June. Plasma ghrelin concentrations were significantly increased through 5 days of fasting during a short-term fast in summer. Over a 24 hour period, ghrelin concentrations increased at night and decreased during the day with drops corresponding to times when squirrels were eating. In January, ghrelin concentrations are low but measurable even while animals are at low body temperature

[2] Published as: Healy, J.E., Ostrom, C.E., Wilkerson, G.K., Florant, G.L., 2010. Plasma ghrelin concentrations change with physiological state in a sciurid hibernator (*Spermophilus lateralis*). Gen Comp Endocrinol 166: 372-378.

(Tb). The reason for the persistence of ghrelin in plasma at this time is unclear, but circulating ghrelin in hibernators may be involved with the control of sleep in these animals. This is the first report of ghrelin concentrations in a non-photoperiodic hibernator. We suggest that ghrelin may be important for the regulation of food intake and the body mass cycle in mammals that hibernate.

III.A.2. *Introduction*

Mammals that hibernate (hibernators) provide unique models for studies on food intake and body condition because of their circannual cycles of obesity and anorexia (Mrosovsky, 1985). Ghrelin is a recently discovered orexigenic hormone which has profound effects on food intake and generally facilitates lipogenesis in mammals (Cummings, 2006; Sangiao-Alvarellos et al., 2009), but there are no published studies on the effects of ghrelin in non-photoperiodic hibernators. Ghrelin is a 28-amino acid protein produced primarily by X/A-like cells in the fundus of the stomach, and in smaller amounts by the intestine, pancreas, and hypothalamus (Dornonville et al., 2001; Hosoda et al., 2000; Kojima et al., 1999; Sakata et al., 2002). The release of ghrelin from the fundus of the stomach occurs in a pulsatory manner, and its circulating levels are dependent on feeding condition (Beck et al., 2003; Toshinai et al., 2001). Ghrelin is known to stimulate growth hormone (GH) secretion and helps regulate energy balance in rodents (Strassburg et al., 2008).

In all mammals studied thus far, fasting results in an elevated concentration of plasma ghrelin (Kinzig et al., 2005; Ortiz et al., 2003; Toshinai et al., 2001). The Siberian hamster (*Phodopus sungorus*) is a rodent that undergoes daily torpor in a seasonal cycle regulated by photoperiod, and as such is not considered a true hibernator.

These animals exhibit a two-fold increase in ghrelin levels during a 48 hour fast, but show no changes in circulating ghrelin levels during a 6 week period of food restriction (Tups et al., 2004). In diurnal mammals, ghrelin levels are highest during nocturnal fasting (Cummings, 2006; Gordon et al., 2005). In rats and humans, plasma ghrelin levels are increased before a meal and decrease immediately following a meal (Beck et al., 2003; Toshinai et al., 2001).

The main physiological effect of ghrelin is an increase in appetite, supported by a food-intake independent increase in adiposity (Depoortere, 2009, Sangiao-Alvarellos et al., 2009). Ghrelin is known to increase food intake when injected intraperitoneally (IP) or intracerebroventricularly (ICV) in rodents (Andersson et al., 2004; Gluck et al., 2006; Keen-Rhinehart et al., 2005; Kojima et al., 1999; Mustonen et al., 2002; Tschop et al., 2000). This increase in food intake is seen in tandem with increased fat mass in most animals studied. Peripherally injected ghrelin increases the respiratory quotient in rodents, indicating an increased dependence on carbohydrate utilization and a decreased fat utilization (Nieminen et al., 2004; Tschop et al., 2000). Ghrelin has other physiological effects, including the promotion of slow wave sleep, which may be important for entry into torpor (Heller & Ruby, 2004; Wiekel et al., 2002).

Hibernators undergo multi-day torpor bouts in winter during which food intake ceases and body temperature (Tb) drops to near ambient temperature (Ta). Golden mantled ground squirrels (GMGS) are diurnal mammals that hibernate and have a robust annual cycle of body mass gain and loss controlled primarily by changes in food intake. During the hyperphagic prehibernation period (late August through mid September in a laboratory setting), GMGS double their food intake and increase body mass nearly 50%,

mostly in the form of fat (Dark, 2005). During this autumnal period, animals also decrease metabolic rate and activity, which promotes fat deposition (Kenagy et al., 1989). GMGS tend to forage in the morning and evening, but are opportunistic feeders (Kenagy et al., 1989). The orexigenic effects of ghrelin may be important in the control of body mass and food intake during this prehibernation period. During the winter hibernation period (Oct. – Mar.), GMGS cease food intake, and many metabolic processes fall to very low rates. During each torpor bout throughout the winter, animals drop Tb to near Ta for several days at a time, and then rewarm to normal Tb of 36ºC for a few hours (become euthermic) before returning to low Tb (usually around 6ºC when Ta is held at 5ºC in a laboratory setting). During these euthermic inter-bout arousals (IBAs), animals do not eat and spend most of the time undergoing sleep (Heller & Ruby, 2004). GMGS emerge from hibernation between March and May, depending on environmental conditions and sex (males emerge earlier than females), and resume normal food intake.

The control of the food intake pathway in hibernators is not fully understood, but hormones such as leptin, insulin, and adiponectin (Florant et al., 2004) are known to play an important role in the seasonal changes in food intake seen in these animals. We hypothesized that ghrelin, which is a highly evolutionarily conserved and orexigenically potent hormone, is likely to be involved in control of food intake in hibernators. In order to test this hypothesis, we carried out several experiments designed to examine changes in plasma ghrelin concentrations in hibernators in different seasons and under different physiological conditions (including a short term fast in summer, a long term fast in winter, and animals undergoing torpor). As an addition to this experiment, we peripherally injected ghrelin into GMGS in June, to test the hypothesis that this would

increase food intake, as it does in most other animals. We hypothesized that a short term fast (1-5 days) in summer would increase plasma ghrelin levels in GMGS. In early autumn (when animals are hyperphagic) it is likely that plasma ghrelin concentrations will be elevated compared to summer levels because increased ghrelin leads to increased food intake. Alternatively, increased food intake during this hyperphagic stage could lead to decreased ghrelin concentrations (due to the effect of a meal decreasing plasma ghrelin). By winter, when animals have ceased feeding and are undergoing torpor bouts, plasma ghrelin concentrations should be low or undetectable. As ghrelin stimulates food intake, an absence of appetite would seem to indicate an absence of ghrelin in the plasma. However, due to the varied physiological effects of ghrelin, it is possible that ghrelin is still circulating in the plasma during hibernation.

III.A.3. *Methods and Materials*

III.A.3.1. Animals and treatment

Forty adult GMGS of both sexes were trapped in Larimer County in the springs and early summers of 2007-2009 and kept in an animal facility at Colorado State University under an approved IACUC protocol. Animals were provided with *ad libitum* food and water and maintained in a warm room (20 ± 2ºC) under natural photoperiod until the beginning of November, when the room temperature was reduced to 5ºC and animals were kept in constant darkness. The lights in the room in which the animals are kept are controlled by a timer which is set to change at the same rate as the external photoperiod. The constant darkness is designed to simulate the entrance of the animal into an underground hibernaculum, where no light is seen from burrow sequestration until emergence from burrow. Animals were separated into the following four groups for

specific ghrelin experiments: (1) Peripheral injection of ghrelin in June (N=8), (2) Short term fast in July (N=18), (3) 24hr ghrelin secretory profile conducted in October (N=5), and (4) Ghrelin levels during the hibernation season (in January) (N=9).

III.A.3.2. Peripheral ghrelin injection

To examine the effects of peripheral ghrelin injection on normophagic GMGS, eight animals were randomly assigned to two groups, one group to be injected intraperitoneally (IP) with ghrelin (N=4), and the other with saline (control, N=4). In June, rat ghrelin (Bachem Corporation) was dissolved in 1 ml sterile diH2O and administered to animals at doses of 10 μg/kg and 50 μg/kg, which is within the range used to elicit an orexigenic response in most rodents (Chen et al., 2004; Keen-Rhinehart et al., 2005). Cumulative food intake (in grams) was measured for each animal for the 6 hours after injections (each animal was injected at 1000 and food intake was measured at 1600). All animals had food and water available *ad lib*.

III.A.3.3. Summer short term fasting

In order to determine if a short term fast would alter plasma ghrelin concentrations, eighteen animals were fasted 0, 1, 3, or 5 days in July (control (fed) N=4, 1-day fast N=5, 3-day fast N=5, 5-day fast N=4) and then euthanized at 1200. Body masses were measured prior to starting the fast, and again on the day of sacrifice to determine how much mass was lost. Change in body mass for control animals was calculated by measuring body mass of animals 5 days before sacrifice and comparing this measurement to that taken at time of sacrifice. Animals were anesthetized with an intramuscular injection of ketamine-acepromazine-xylazine (75%-20%-5%). Blood

samples were collected by cardiac puncture, were centrifuged, and plasma was removed and stored at -80ºC until analysis.

III.A.3.4. 24hr ghrelin circulation profile

In mid-October (at the end of the GMGS hyperphagic period), five animals were catheterized in the jugular vein under sterile conditions. An intramuscular injection of ketamine-acepromazine-xylazine was used to initially anesthetize the animals, and then deep anesthesia was maintained using a 2-3% isoflurane gas. The internal jugular vein was isolated through a 1-1.5 cm incision in the ventral neck. A sterile 0.6mm diameter polyethylene tapered catheter was inserted into the vein and extended into the caudal aspect of the vessel (toward the heart) approximately 1.5 cm. The catheter was secured into the vein and surrounding musculature with 4-0 absorbable monofilament suture. The internal jugular vein cranial to the catheter-insertion site was ligated using the same suture material. A 2 mm skin incision was made on the midline of the back between the two scapulae and a subcutaneous tunnel approximately 2 mm in diameter was made between this incision and the ventral neck incision using a surgical trocar. The free end of the catheter was then passed through this tunnel from its position in the neck area to the dorsal incision site. The skin incision on the back was closed and the free end of the catheter (injection port) was secured into place using 3-0 sterile monofilament nylon suture. The neck incision was closed with the same nylon suture. Animals recovered from surgery on heating pads and were administered 0.05 ml buprenorphine twice a day for three days for post-surgical analgesia.

The animals were allowed to recuperate from surgery for three days, and then 0.3 ml of blood was drawn through each animal's catheter every two hours for 24 hours

under a 14 L: 10 D photoperiod with food and water available *ad libitum*. Blood collection was performed as non-invasively as possible during dark hours. A red safe light (3-5 lux) was used when entering the room, and blood was withdrawn from catheters while squirrels were allowed to remain in their nests. Blood samples were centrifuged and plasma was removed and stored at -80ºC. Red blood cells were re-suspended in 0.3 ml sterile heparinized saline and reinjected into each animal through its catheter. Animals were remotely monitored via video camera and the number of times animals fed per hour was recorded. Three weeks later, the animals were again anesthetized and the indwelling catheters were removed.

III.A.3.5. Ghrelin levels during hibernation

For the fourth experiment, five animals undergoing torpor in January (at LTT (6-9ºC)) were sacrificed by decapitation at 1200 and blood samples were collected. Low Tb was confirmed by thermocouple readings of skin, mouth, blood, and body cavity temperatures. Blood samples were collected by cardiac puncture, were centrifuged, and plasma was removed and stored at -80ºC until analyzed. Stomachs were dissected out and examined for traces of food.

To determine euthermic ghrelin levels in January, four animals were aroused from torpor. After animals became completely euthermic (Tb>30ºC, moving freely around cage) they were anesthetized for blood sampling. Blood samples were collected by cardiac puncture, were centrifuged, and plasma was removed and stored at -80ºC until analysis. Animals were then allowed to return to torpor.

III.A.3.6. Validation and use of assay

All plasma ghrelin concentrations were determined using an EIA assay (measuring total ghrelin) from Phoenix Pharmaceuticals (EK-031-31). The assay was validated using a GMGS serial plasma dilution curve. Samples were assayed in duplicate and diluted as follows: 50µl sample: 0µl assay buffer (5:0), 30µl sample: 20µl assay buffer (3:2), 20µl sample: 30µl assay buffer (2:3), and 10µl sample: 40µl assay buffer (1:4) (results shown in Figure III.A.1).

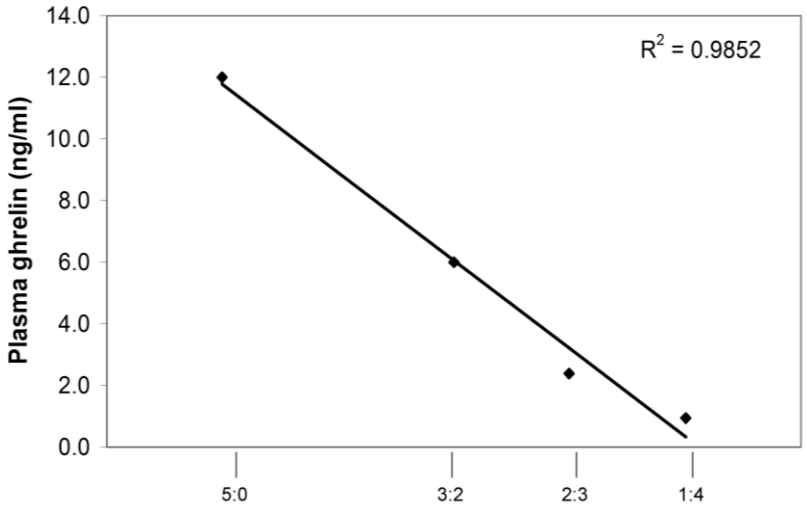

Figure III.A.1: Validation of total ghrelin EIA assay: dilution curve for GMGS (sample diluted at 5:0, 3:2, 2:3, and 1:4 with assay buffer) (From Healy et al., 2010)

The sensitivity of this assay is 0.08 ng/ml; intra-assay variation is <5% and inter-assay variation is <14%. All statistical analysis was performed using SAS 9.1. A Student *t*-test was used to evaluate mean differences, which were considered significant if $p<0.05$. A single-factor ANOVA was used to evaluate differences between hours in the 24 hour circulating ghrelin experiment. When ANOVA indicated significant variations, the Student-Newman-Keuls test was used to compare hourly means. All differences were considered statistically significant at $p<0.05$.

III.A.4. *Results*

III.A.4.1. Effect of peripheral ghrelin injection on summer food intake

Our dose titration experiment showed that an IP injection of rat ghrelin (at either 10 or 50 µg/kg) stimulated food intake in *ad libitum* fed GMGS. There was no difference in food intake response between the 10 and the 50 µg/kg groups, so they were analyzed together for statistical purposes. Animals that received IP ghrelin injections significantly increased food intake compared with animals injected IP with saline (p=0.026). Animals injected with ghrelin ate an average of 7.9 grams over a period of six hours after injections whereas saline-injected controls ate an average of 3.6 grams over the same time period (Figure III.A.2).

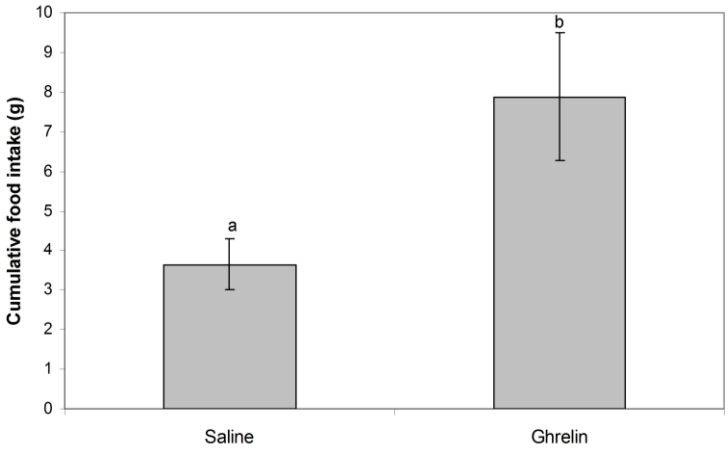

Figure III.A.2: Mean cumulative food intake (in grams) for the six hours after peripheral saline and ghrelin injections in June (control N=4, ghrelin N=4); bars with different letters are statistically different (From Healy et al., 2010)

III.A.4.2. Ghrelin levels after a short term fast in summer

Animals that had been fasted for one day had significantly elevated (p=0.043) plasma ghrelin concentrations compared with controls (5.44 ng/ml vs. 3.60 ng/ml respectively) (Figure III.A.3.b).

Animals fasted for three and five days also had elevated ghrelin concentrations that were significantly higher than controls (p=0.049), but not significantly different from animals fasted for one day. Figure III.A.3.b illustrates the average change in body mass caused by each fasting condition. Control animals gained mass while animals under each of the fasting conditions lost progressively and significantly more mass.

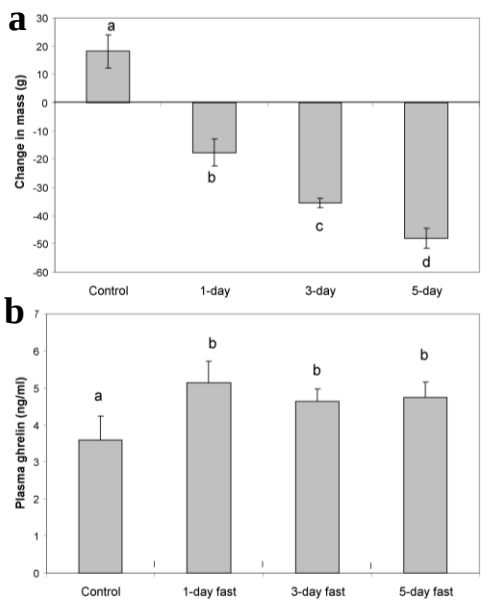

Figure III.A.3.a: Mean change in body mass with fasting ± SEM during short term fast in July (control N=4, 1-day N=5, 3-day N=5, 5-day N=4). Different letters are statistically different ($p<0.05$); **3.b:** Mean ghrelin concentrations ± SEM in short-term fast in July. Letters a & b are statistically different ($p<0.05$) (From Healy et al., 2010)

III.A.4.3. 24hr pattern of circulating ghrelin in October

The 24hr plasma ghrelin concentrations are illustrated in Figure III.A.4.b and plotted against observed feeding times for catheterized squirrels. Plasma ghrelin increased within the first 2 hours of the dark period (2100-2300), decreased through the night (2100-0700), and then rose again prior to lights on (0700); these two peaks were statistically higher than preceding points at $p<0.05$.

When lights came on (0700), animals were observed eating and ghrelin concentrations were decreased, remaining at a low level from 0900 until 1500 after which ghrelin rose slightly just before the dark period began. The mean plasma ghrelin concentration during the dark period was 6.6 ng/ml which was significantly greater than

the mean ghrelin concentration during the light period (5.3 ng/ml) (p=0.019). Figure III.A.4.a illustrates the average food intake for the catheterized GMGS during the month of October before, during, and after the catheterization experiment. Animals were eating between 8-12 grams/day at the end of their hyperphagic period (late August to mid September in the lab).

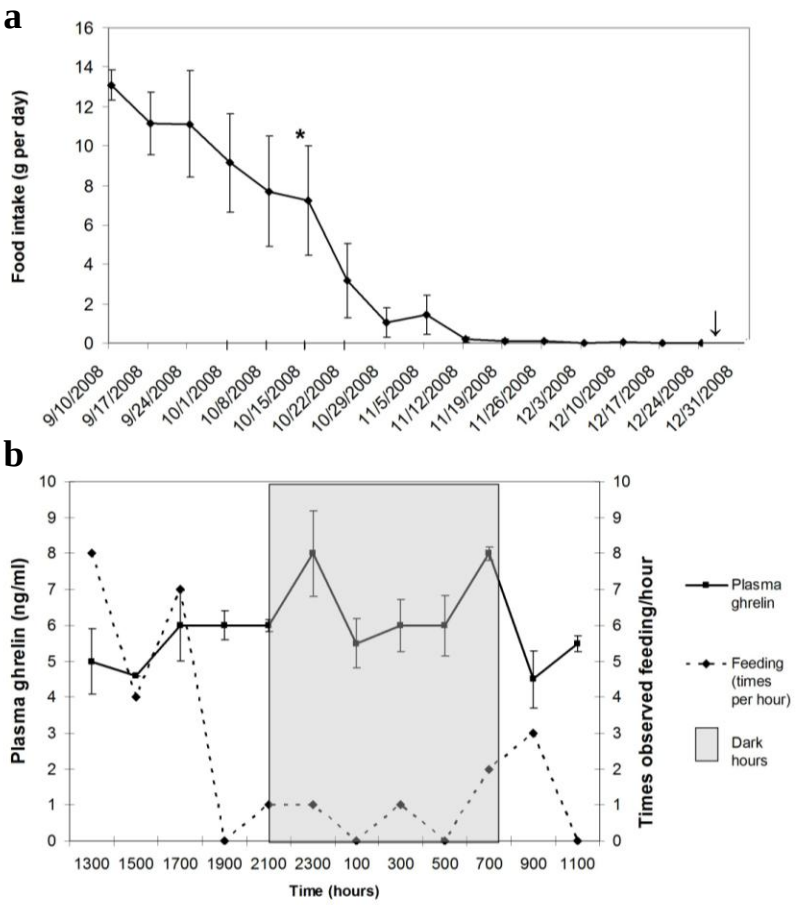

Figure III.A.4.a: Mean food intake ± SEM per day from September to January 2008-2009 (N=5), including times of 24-hour ghrelin secretion experiment and January ghrelin experiment *=animals catheterized for 24 hour ghrelin experiment; ↓=animals sacrificed for January ghrelin experiment; **4.b:** 24-hour plasma ghrelin secretory pattern and times observed feeding/hour in GMGS—shaded area denotes dark hours (lights out); each point on ghrelin line represents the mean ± SEM for each time period; each point on feeding line represents the total number of times animals were observed feeding for each time period (N=5) (From Healy et al., 2010)

III.A.3.4. Ghrelin levels during hibernation

In January, plasma ghrelin levels were significantly higher (p=0.01) in euthermic animals (animals undergoing an interbout arousal (IBA)) than in animals hibernating at low tissue temperature (LTT) in January (Figure III.A.5). Food was present in the animals' cages throughout the winter, but weekly measurement of food intake indicated that animals were not eating during this time (Figure III.A.4.a). The stomachs of animals euthanized at LTT were examined and found empty of food.

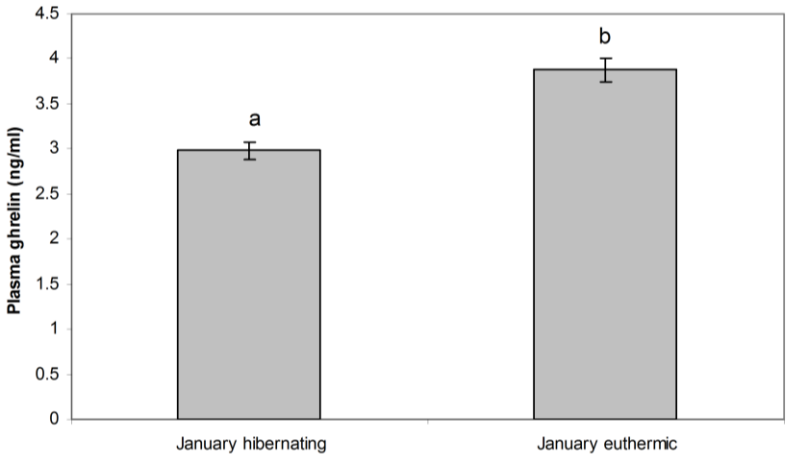

Figure III.A.5: Mean ghrelin concentrations ± SEM in animals at low tissue temperature in January (LTT) (N=5) and in animals euthermic in January (N=4). Letters a & b are statistically different ($p<0.05$) (From Healy et al., 2010)

III.A.5. *Discussion*

This is the first report of plasma ghrelin concentrations in an obligate hibernator. Circulating ghrelin has been shown to promote an orexigenic response in all mammals studied thus far (Depoortere, 2009). We found that GMGS responded to peripheral ghrelin injections with a significant increase in food intake, in concurrence with studies on non-hibernating rodents (Gluck et al., 2006; Keen-Rhinehart et al., 2005; Nieminen et al., 2004; Tschop et al., 2000). Ghrelin receptors are located in the pituitary and in multiple nuclei of the hypothalamus, and when bound by ghrelin stimulate particular food intake pathways. The activation of the ghrelin receptor leads to the phosphorylation and

concomitant activation of adenosine-monophosphate protein kinase (AMPK) (Anderson et al., 2004), which causes an increase in food intake and fatty acid synthesis via the orexigenic peptides neuropeptide Y (NPY) and agouti-related protein (AGRP) (Chen et al., 2004), and down-regulation of the anorexigenic pro-opiomelanocortin (POMC) (Anderson et al., 2004). It has been proposed that peripheral ghrelin and hypothalamic ghrelin take part in two parallel outputs (stimulation of food intake and control of sleep-wake cycle) (Szentirmai et al., 2007a,b), and that a baseline ghrelin concentration must be met for stimulation of hunger (Schuessler et al., 2006).

During short fasts in summer, ghrelin levels increased significantly ($p<0.05$) between control animals and one, three, or five day fasted animals, consistent with previous studies in rodents (Tups et al., 2004; Toshinai et al., 2001; Ortiz et al., 2003; Mustonen et al., 2002). Ghrelin levels did not change significantly between days of fasting; this result lends support to the hypothesis that ghrelin is a short-acting hormone (Cummings, 2006; Tups et al., 2004). Others, however, have hypothesized that ghrelin can act over the long term (Epelbaum et al., 2009; Strassburg et al., 2008), and some of our results support this hypothesis. Ghrelin levels in mid October (when animals are nearing the end of their hyperphagic period) are significantly higher than those measured at the same time of day in July (5.6 ng/ml vs. 3.6 ng/ml respectively, $p<0.05$), and both summer and autumn levels are higher than those seen in hibernating GMGS (when animals are not eating). These results indicate that ghrelin secretion may be altered by season, and therefore could play a role in the seasonal changes in food intake seen in hibernators. Scrimgeour et al. (2008) provide evidence that ghrelin may reflect long term energy balance, acting as a feedback signal to the hypothalamus to defend an optimum

body mass by controlling food intake. If a hibernator's energy stores are depleted to a sufficiently low level during torpor, it will arouse and attempt to eat to make up the difference (Mrosovsky et al., 1970). These arousals to euthermia are energetically expensive (incurring up to 86% of the energetic cost of hibernation (Wang, 1978)), and it behooves an animal to arouse as little as possible during the hibernation season (Humphries et al., 2002). By maintaining plasma ghrelin at a low level, the urge to eat is suppressed, removing one possible stimulus to arousal. The ability to maintain low plasma ghrelin levels during the hibernation season may be important in regulating the delicate energy balance maintained by a hibernating mammal.

Ghrelin is known to facilitate torpor bouts in food deprived mice, and this action appears to be mediated by the arcuate nucleus (Gluck et al., 2006). Peripheral ghrelin administration in fasted mice deepened already occurring torpor bouts by several degrees. This may occur through the stimulation of the release of NPY by ghrelin, since ICV injected NPY is known to cause a torpor-like hypothermia in Siberian hamsters (Dark & Pelz, 2008). As torpor in mice is initiated through fasting, ghrelin levels in torpid mice are expected to be high. Conversely, GMGS cease food intake (fast voluntarily) some time before entering hibernation. Since the drive to eat is reduced, we expected that ghrelin levels during hibernation would be very low or undetectable.

Interestingly, although GMGS do not eat during the hibernation season, ghrelin was still present in the plasma of our experimental animals hibernating at low tissue temperature. Since ghrelin is usually strongly orexigenic, its presence in a time of aphagia suggests that it may be fulfilling other physiological processes, perhaps stimulating non-rapid eye movement (NREM) sleep. The long-accepted paradigm is that

torpor is entered through sleep—as brain temperature falls, NREM sleep predominates and REM sleep decreases (Walker et al., 1977). Unfortunately, it is difficult to ascertain the differences in hibernating sleep states because electrical processes slow as temperature drops (Heller & Ruby, 2004; Deboer, 1998). Since neurons in certain parts of the brain show specific EEG patterns for each sleep state, there has been some success at differentiating periods homologous to NREM and wakefulness at brain temperatures down to 14ºC (Krilowicz et al., 1988). The effects of ghrelin on sleep are controversial and seem to vary by species (for a review see Steiger, 2006). Ghrelin is known to increase during the first hours of sleep and promotes slow wave sleep (SWS) in humans and mice (Obal et al., 2003; Weikel et al., 2003). The low concentrations of plasma ghrelin measured in torpid and euthermic GMGS may be functioning to facilitate NREM sleep during torpor bouts and interbout arousals (IBAs). This could be tested by establishing a ghrelin secretion profile for a torpor bout and associated IBA and recording sleep patterns at the same time, followed by injecting ghrelin to measure the effect on sleep patterns.

In previous studies on diurnal mammals, plasma ghrelin levels stay fairly high throughout the dark period compared with daytime levels, but rise sharply just before waking (Cummings et al., 2001). The 24hr ghrelin secretion profile for our GMGS was determined at the end of the hyperphagic period (mid October), and shows that mean plasma ghrelin levels were significantly higher during the dark period (when GMGS had gone the longest without eating) than during the light period ($p<0.05$). This is mainly due to two peaks—one occurring shortly after the lights were turned off, and the other occurring immediately prior to lights being turned on. It is possible that the first peak

observed in plasma ghrelin was involved in stimulating slow wave sleep, and the second in stimulating feeding. Several recent articles have proposed that plasma ghrelin must reach a threshold level to stimulate food intake, and that smaller increases in plasma ghrelin may be involved in control of sleep (Steiger, 2007; Szentirmai et al., 2007b).

In our experiment, plasma ghrelin concentrations dropped after squirrels ate at the start of light period. In previous studies on rats, which are nocturnal, ghrelin levels were highest during light hours and dropped during dark hours when animals were awake (Bodosi et al., 2004; Murakami et al., 2002; Tolle et al., 2002). The secretion profiles illustrated in these studies show a common pattern; our secretion profile for GMGS shows a similar pattern when adjusted for their diurnal lifestyle (plasma ghrelin levels were highest when animals had gone the longest without eating—early morning for GMGS, late evening for nocturnal animals).

Ghrelin may be important for regulation of the prehibernation food intake cycle in hibernators, and is possibly linked with the cyclic obesity shown in these animals. Ghrelin is thought to be primarily a short-term acting hormone, but recent studies (Strassburg et al., 2008), including ours, suggest that it may have long-term effects on food intake and body mass. Further research is necessary to elucidate the various physiological effects of ghrelin, but it may be an important hormone in regulating the food intake and circannual body mass cycle of hibernators.

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III.B. Peripheral ghrelin stimulates feeding and positive energy balance in a sciurid hibernator

III.B.1. *Abstract*

Hibernators exhibit a robust circannual cycle of body mass gain and loss primarily mediated by food intake, but the pathways controlling food intake in these animals have not been fully elucidated. Ghrelin is an orexigenic hormone that increases feeding in all mammals studied so far, but has not until recently been studied in hibernators. In other mammals, ghrelin stimulates feeding through phosphorylation and activation of AMP-activated protein kinase (AMPK). Activation of AMPK phosphorylates and deactivates acetyl Co-A carboxylase (ACC), a committed step in fatty acid synthesis. In order to determine the effects of exogenous ghrelin on food intake and metabolic factors (i.e. non-esterified fatty acids (NEFAs), and hypothalamic AMPK and ACC) in hibernators, ghrelin was peripherally injected into ground squirrels in all four seasons. Changes in food intake and body mass were recorded over a 2-6 hour period post injections, and squirrels were euthanized. Brains and blood were removed, and Western blots were performed to determine changes in phosphorylation of hypothalamic AMPK and ACC. A colorimetric assay was used to determine changes in concentration of serum NEFAs. We found that food intake, body mass, and locomotor activity significantly increased with ghrelin injections versus saline-injected controls, even in animals injected during their aphagic winter season. Injected ghrelin was correlated with increased phosphorylation of AMPK, but didn't have an effect on ACC in winter. Ghrelin-injected animals also had increased levels of serum NEFAs than saline controls. This study is the first to show an effect of injected ghrelin on a hibernator.

III.B.2. *Introduction*

Hibernation is an energy-saving life history strategy employed by animals in many different genera. Mammals that hibernate (hibernators) undergo multi-day torpor bouts in winter during which food intake ceases and body temperature (Tb) drops to near ambient temperature (Ta). The golden-mantled ground squirrel (GMGS, *Callospermophilus lateralis*) is a diurnal hibernator with a robust annual cycle of body mass gain and loss primarily due to changes in food intake. During the hyperphagic prehibernation period (late July-September), GMGS double their food intake and increase body mass nearly 50%, mostly in the form of fat (Dark, 2005). During this autumnal period, animals also decrease metabolic rate and activity, which aids in building fat stores (Kenagy et al., 1989). During the winter hibernation period (October-March), GMGS cease food intake, and metabolic processes drop to very low rates. During each torpor bout throughout the winter, animals drop Tb to near Ta (usually around 5ºC) for several days at a time, and then rewarm to a normal Tb of 36ºC for a few hours (become euthermic) before returning to low Tb. During these euthermic inter-bout arousals (IBAs), animals do not eat and spend most of the time undergoing sleep (Torke and Twente, 1977; Heller and Ruby, 2004). Some male GMGS emerge from hibernation in late February, but females tend to remain torpid through mid-March. During the spring and summer months, GMGS reproduce and are euthermic and normophagic (Dark, 2005). GMGS tend to forage in the morning and evening, but are opportunistic feeders (Kenagy et al., 1989).

Little is known about the physiological controls of food intake in hibernators. Ghrelin is a recently discovered orexigenic gut/brain peptide that has various

physiological effects; its effects on food intake and lipogenesis are well documented (Cummings, 2006; Strassburg et al., 2008), but it also appears to have effects on animal behavior that have not been clearly elucidated. Some of the evidence is apparently contradictory. For instance, in a 2006 study, ghrelin injected into lateral cerebral ventricles of rats increased exploratory behavior and spontaneous locomotor activity in rats (Jaszberenyi et al., 2006), contrary to the results of a 2005 study during which ghrelin injected into lateral cerebral ventricle of rats decreased spontaneous locomotor activity while increasing food intake (Castaneda et al., 2005). In the latter study, the increase in food intake was immediate, while the decrease in activity took some time to appear. This suggests that ghrelin first increases food seeking behavior, but later increases energy saving behavior (Castaneda et al., 2005). Ghrelin injected peripherally in Siberian hamsters (*Phodopus sungorus*) increased foraging behavior, food hoarding, and food intake, but had no effect on spontaneous locomotor activity (Keen-Rhinehart and Bartness, 2005).

The ghrelin receptor, growth hormone secretogogue receptor 1 (GHSR1), is found in various areas of the central nervous system. The majority of expression is in the hypothalamus, with some expression in the cerebral cortex and the dorsal vagal complex of the medulla oblongata (Kojima et al., 1999; Cowley et al., 2003; Hou et al., 2006). In non-hibernators, one action of ghrelin is to stimulate phosphorylation and activation of AMP-activated protein kinase (AMPK) in the hypothalamus, which in turn phosphorylates and deactivates acetyl Co-A carboxylase (ACC), a committed step in fatty acid synthesis (Andersson et al., 2004, Kola et al., 2005, Kohno et al., 2008). There is

some evidence that modification of these enzymes is required for the orexigenic effects of ghrelin (Kohno et al., 2008, Lage et al., 2010).

In addition to increasing food seeking behavior, ghrelin has been shown to alter other behaviors. Ghrelin injected intraperitoneally (IP) and intracerebroventricularly increased food intake and anxiogenic behavior in rats (Asakawa et al., 2001; Carlini et al., 2002). Similarly, a study by Kodomari et al (2009) found that offspring of ghrelin-treated female mice exhibited increased stress behavior (measured as increased movement from the center of an open field).

We have recently measured ghrelin levels in the serum of hibernating and winter euthermic GMGS, and found that ghrelin was still circulating in the blood even at low tissue temperature (Healy et al., 2010). In order to determine the effects of ghrelin on hibernators, we hypothesized that peripherally injected ghrelin would cause an increase in food intake and activity in GMGS during the three seasons during which they are euthermic and eating, but that ghrelin injected into aphagic GMGS in the winter season would have no effect. Since ghrelin's orexigenic effect may be dependent on the modification of AMPK and ACC, we expected that hypothalamic concentrations of these peptides would be low in the control animals, but would increase in reaction to injected ghrelin. We also hypothesized that since injected ghrelin should increase positive energy balance within the animal by stimulating food intake, animals injected with ghrelin would have an increased level of serum non-esterified fatty acids (NEFAs) when compared with saline-injected controls.

III.B.3. *Methods and Materials*

III.B.3.1. Animals

Adult GMGS of both sexes were trapped in Larimer County in the spring and early summers of 2008-2009 and kept in an animal facility at Colorado State University under an approved IACUC protocol. Animals were provided with cotton for nesting material, *ad libitum* food (Harlan Teklad 8640; Madison, WI, USA) and water, and maintained in a warm room (20ºC) under natural photoperiod (Paragon Sun Tracker EC72ST; Invensys Controls, Carol Stream, IL, USA) until the beginning of November, when the temperature was reduced to 5ºC and animals were kept in constant darkness (to mimic natural burrow conditions) for the remainder of the hibernation season.

III.B.3.2. Ghrelin injection experiment

Animals were randomly assigned to two groups, one group to be injected IP with ghrelin (N=18), and the other with saline (control, N=18). These two groups were then broken down into four smaller groups each: Summer (animals had been normophagic and consistently euthermic for 2 months, N=4/group), Autumn (animals were hyperphagic, at twice their normal food intake, N=4/group), Winter (animals had been aphagic and heterothermic for 2 months, N=7/group), and Spring (animals had been euthermic and normophagic for less than 1 month, N=3/group). Mouse/rat ghrelin (Bachem Corporation, H-4862) was dissolved in 1 ml sterile saline and used immediately. On one day of each season, 1-ml syringes with 25 gauge needles were prepared with either 50µl saline or 50µl ghrelin solution (at a dose of 50µg/kg, which is within the range used to elicit an orexigenic response in most rodents (Chen et al., 2004; Keen-Rhinehart and Bartness, 2005)). GMGS were weighed and were injected IP with saline or ghrelin

solution. Cumulative food intake (in grams) was measured for each animal at 2 and 6 hours after injections (each animal was injected at 1000 hrs. and food intake was measured at 1200 and 1600 hrs.). All animals had food and water available *ad libitum*. Behavior was remotely monitored via video camera for 6 hours following the injections; at the end of this period, food intake was measured and animals were weighed again.

III.B.3.3. Hypothalamic dissection

Animals in the summer group were treated as described above, with the exception that behavior, food intake, and body mass were monitored for only two hours. Two hours after injections, animals were anesthetized with an intramuscular injection of ketamine-acepromazine-xylazine (75%-20%-5%). Blood samples were collected by cardiac puncture. Samples were allowed to coagulate before being centrifuged, and serum was removed and stored at -80ºC until analysis. Animals were euthanized by decapitation; brains were removed, flash-frozen in 2-methylbutanol and stored at -80ºC until use. Animals in the winter group were aroused to euthermia at 0700 hrs. and allowed to regain normal euthermic function for three hours. Animals were then euthanized as described above. Hypothalami from all brains were dissected out (using stereotaxic ground squirrel brain atlas by Joseph et al. (1966)) and homogenized in 0.5ml lysis buffer with protease inhibitor cocktail, centrifuged, and the supernatant removed. Homogenates were frozen at -80ºC until assayed.

III.B.3.4. Neuropeptide analysis

Protein concentration was determined by BCA assay and Western Blots were performed on hypothalamus homogenate. Briefly, sample proteins were separated by SDS-PAGE and transferred to nitrocellulose. Equal amounts of protein were added to

each gel, as confirmed by β-actin. The membranes were blocked in TBS with 5% milk powder and incubated overnight on an orbital shaker at 4ºC in primary antibody (diluted 1:1000 for phosphorylated AMPK (pAMPK), total AMPK, phosphorylated ACC (pACC), total ACC, and β-actin). Antibodies were obtained from Cell Signaling (Phospho-AMPKα (Thr172) Rabbit mAb #2531, AMPKα (23A3) Rabbit mAb #2603, Phospho-Acetyl-CoA Carboxylase (Ser79) Antibody #3661, Acetyl-CoA Carboxylase (C83B10) Rabbit mAb # 3676, β-actin Antibody # 4967). After washing in TBST, membranes were incubated at room temperature for 1 hr in HRP-conjugated secondary antibody (1:2000) and HRP-conjugated anti-biotin antibody (1:1000). After further washing in TBST, membranes were developed by chemiluminescence (Amersham ECL Plus from GE Healthcare), and imaged on a STORM. Protein expression was quantified using ImageQuant (GE Healthcare) and normalized against β-actin.

III.B.3.5. Serum metabolites and hormones

Serum NEFA concentrations were determined by a commercially available colorimetric assay from Wako Chemicals (HR Series NEFA-HR (2)), using a BioTek Synergy HT microplate reader. Serum ghrelin was measured with an enzyme immunoassay from Phoenix Pharmaceuticals (EK031-31) as previously described (Healy et al., 2010).

III.B.3.6. Activity analysis

A small video camera (Sony Handycam DCR-HC20) was set approximately 6 feet from animal cages to stream directly into a laptop computer capturing the 6 hours after injections. After animal sacrifices, videos were analyzed for behavioral activity for each squirrel (only the initial two hours of post-injection behavior are reported here).

Behaviors were categorized as inactive (animal in nest, not moving), non-specific activity (animal visibly moving around either in nest or outside of nest), nesting (animal moving cotton around cage or fluffing cotton in nest), feeding (animal observed putting food in mouth or keeping head in food bowl >5 seconds), grooming (animal observed licking, scratching, etc. either in or outside of nest), or drinking (animal's mouth on water sipper or head in water bowl). Behavior in all videos was analyzed by one person to minimize variation.

III.B.3.7. Statistics

Statistics were performed using Graph Pad Prism 5. Differences in food intake, body mass, NEFAs, and neuropeptide levels between control and experimental groups were determined by Student's t-test. Behavioral differences between seasons and experimental groups were tested using a 2-way ANOVA with Bonferroni post-test. All results were considered significant at $p \leq 0.05$.

III.B.4. *Results*

III.B.4.1. Food intake

Animals injected with ghrelin significantly increased food intake compared with saline controls during all four seasons (Figure III.B.1). In spring, GMGS ate an average of 1.5g over a two hour period when injected with saline, but 4g per 2 hours when injected with ghrelin. In summer, saline-injected animals averaged 1.25g over a two hour period, while ghrelin-injected animals ate 6.5g. In autumn, animals injected with saline ate about 2g over 2 hours, while ghrelin-injected animals averaged 4.5g over the same time period.

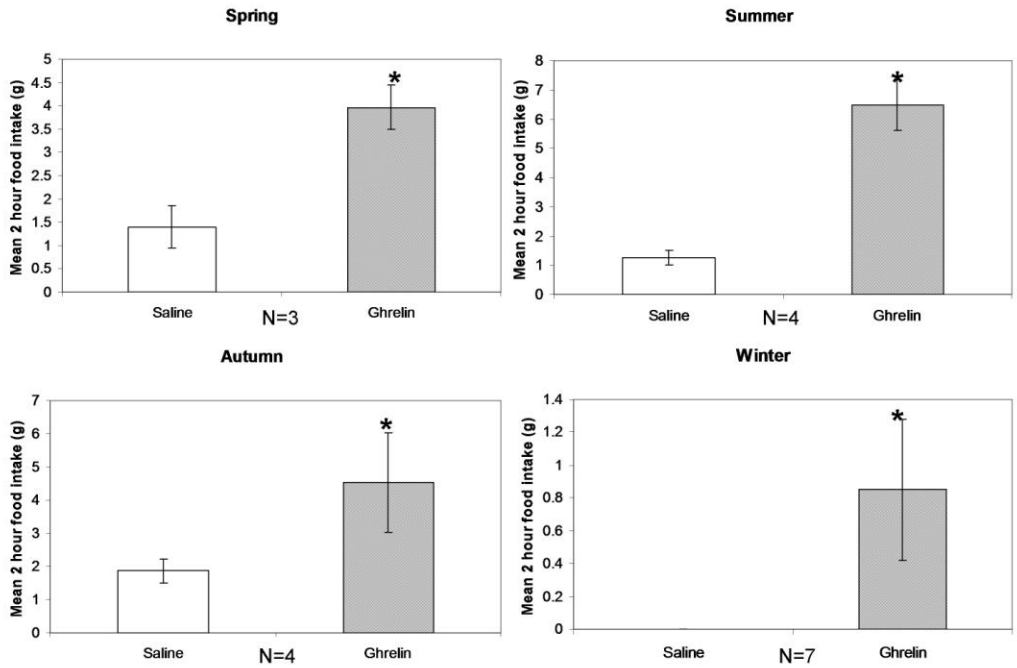

Figure III.B.1: Average cumulative food intake after saline or ghrelin injections by season; * = significantly increased ($p \leq 0.05$) over saline

In winter, when hibernators are normally aphagic, saline-injected animals did not eat, but five of seven ghrelin-injected animals initiated food intake, eating a mean of 0.85g over a 2 hour period (Figure III.B.1).

III.B.4.2. Body mass

Animals injected with ghrelin either gained more mass or lost less mass than animals injected with saline in all seasons (Table 1). These increases were not always significant, and were dependent on the ratio of the amount of food eaten to the total time active.

Table 1
Changes in body mass 2 hours after injections of ghrelin or saline

Treatment	Spring	Summer	Autumn	Winter
Saline	1.11 ± 0.23	-2.96 ± 0.34	0.50 ± 0.59	0 ± 0
Ghrelin	2.52 ± 0.92	-0.89 ± 0.47*	3.01 ± 1.17*	0.33 ± 0.18
P value	0.07	0.006	0.05	0.06

Mean change in body mass per group ± SEM 2 hours post injection. Positive numbers indicate mass gain, and negative numbers indicate mass loss. *Differences between treatments by season are considered significant at $p \leq 0.05$.

III.B.4.3. Neuropeptides

Western blots were performed to determine expression of total and phosphorylated forms of AMPK and ACC. In summer, ghrelin-injected animals had significantly increased levels of both total and phosphorylated AMPK and ACC when compared with saline controls (Figure III.B.3). Winter animals injected with ghrelin significantly increased the phosphorylated (activated) form of AMPK compared with saline controls (Figure III.B.5). There was no effect of ghrelin on total AMPK or phosphorylated or total ACC in winter animals.

III.B.4.4. Serum ghrelin and NEFAs

Animals injected with ghrelin had increased serum ghrelin concentrations when compared with saline control, both in summer (Figure III.B.2) and winter (Figure III.B.4).

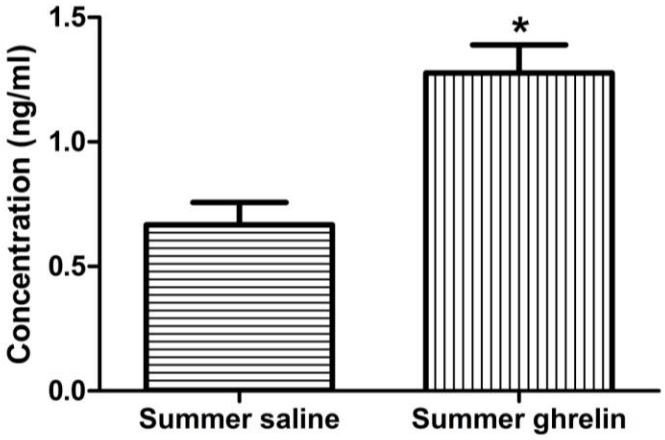

Figure III.B.2: Effect of summer saline and ghrelin injections on serum ghrelin levels; * = ghrelin statistically different from saline ($p \leq 0.05$)

Ghrelin-injected animals also showed higher NEFA concentrations than saline-injected controls Figure III.B.6).

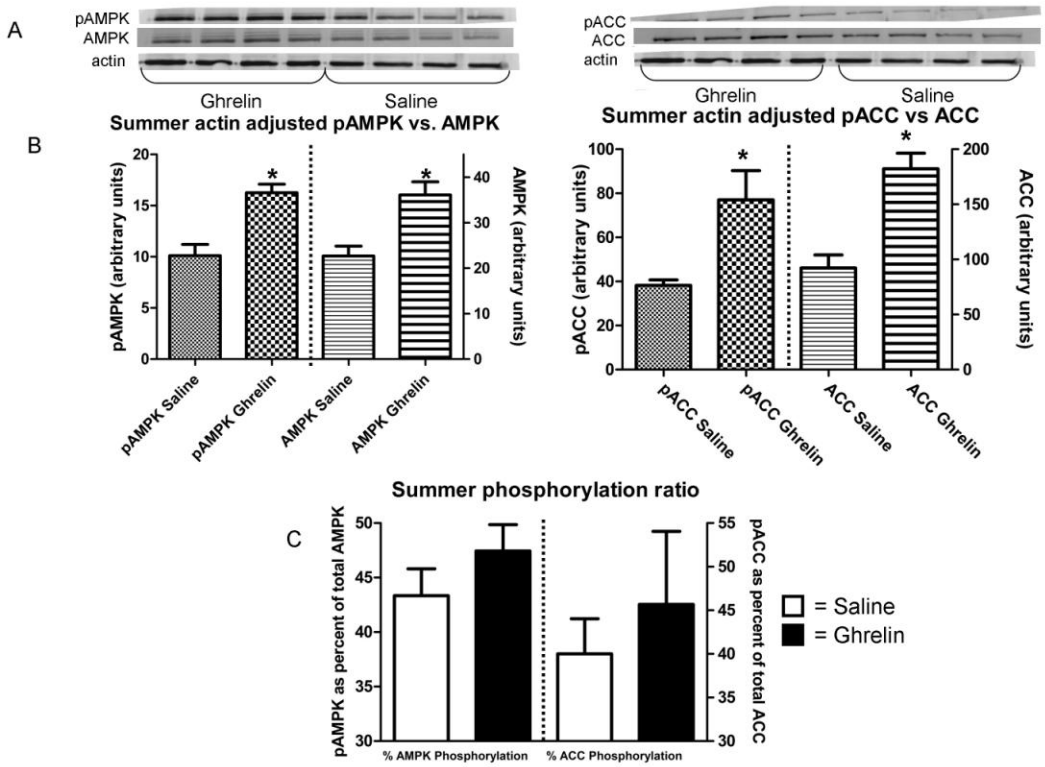

Figure III.B.3: (A) Representative Western blots for hypothalamic expression of pAMPK/AMPK, pACC/ACC, and actin (N=4 per group)
(B) Effect of summer saline and ghrelin injections on hypothalamic AMPK and ACC (total and phosphorylated)
(C) Ratio of phosphorylated to total AMPK and ACC; * = ghrelin significantly elevated over saline ($p \leq 0.05$)

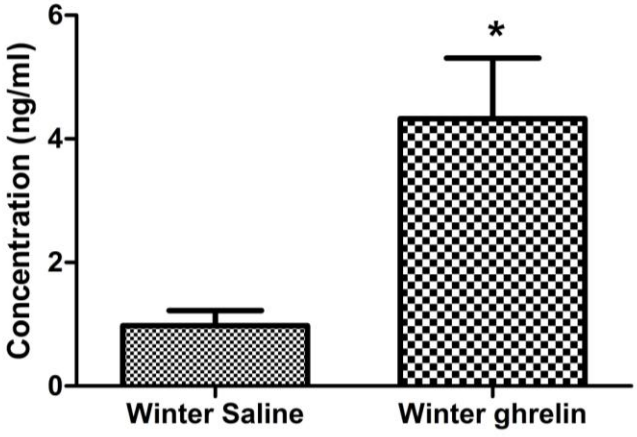

Figure III.B.4: Effect of winter saline and ghrelin injections on serum ghrelin concentrations
* = ghrelin significantly elevated over saline ($p \leq 0.05$)

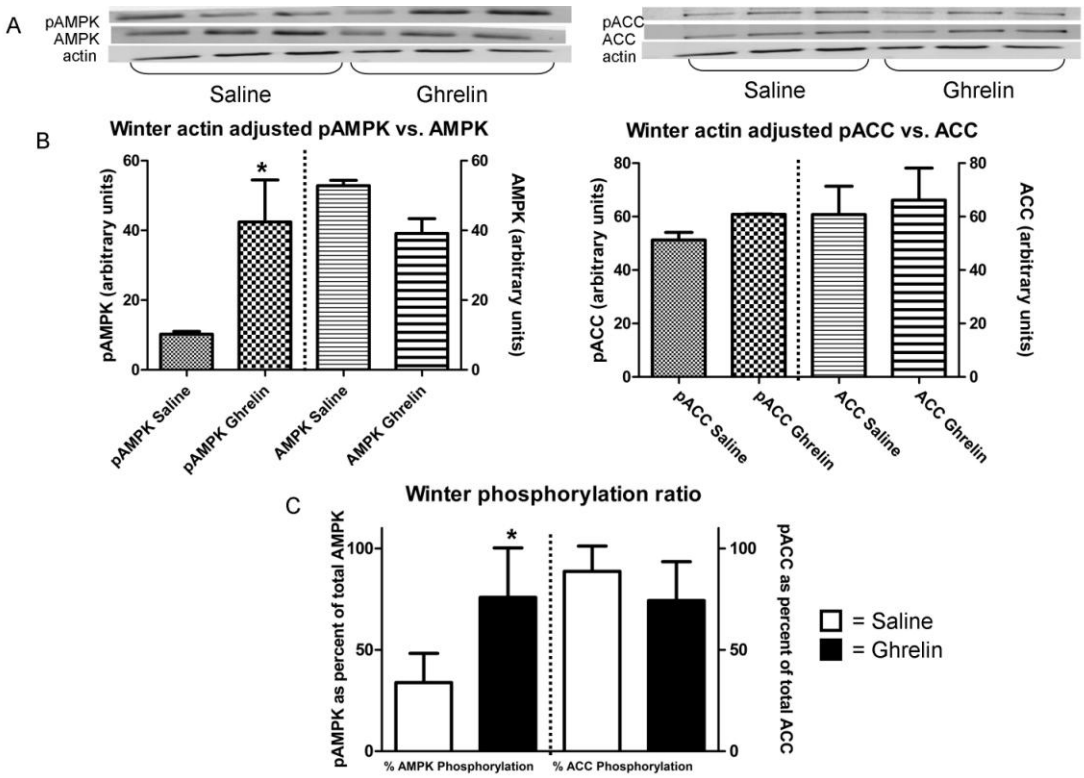

Figure III.B.5: (A) Representative Western blots for hypothalamic expression of pAMPK/AMPK, pACC/ACC, and actin (N=3 per group)
(B) Effect of winter saline and ghrelin injections on hypothalamic AMPK and ACC (total and phosphorylated)
(C) Ratio of phosphorylated to total AMPK and ACC; * = ghrelin significantly elevated over saline ($p \leq 0.05$)

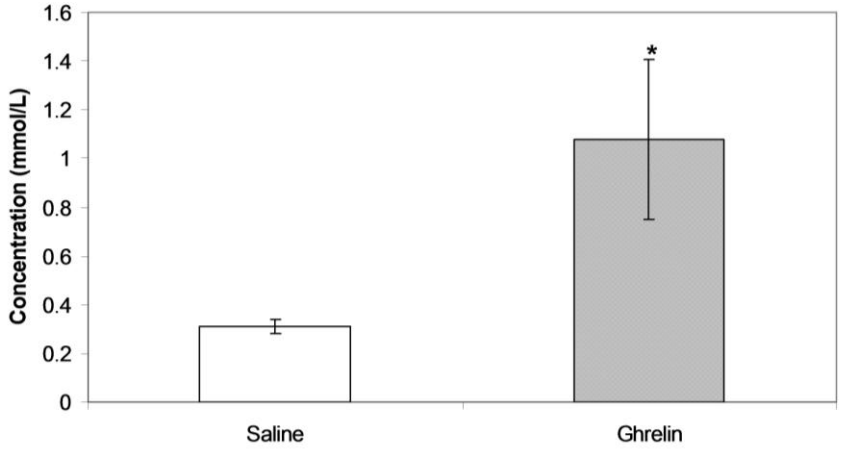

Figure III.B.6: Effect of winter saline and ghrelin injections on serum NEFAs (N=4 per group); * = ghrelin significantly elevated over saline ($p \leq 0.05$)

III.B.4.5. Behavior

Videos recording the two hours after injections were analyzed to determine what effects peripherally injected ghrelin would have on behavior. In general, animals injected with ghrelin were more active than those injected with saline, especially in feeding, grooming, and nesting behaviors, but these differences were not always statistically significant (Table 2).

Table 2
Behavioral changes induced by injection of ghrelin

Treatment	Inactivity	N.S. Activity	Feeding	Grooming	Nesting	Drinking
Saline						
Summer	6971 ± 6971	65.75 ± 65.75	37.5± 37.5	118± 118	7.5± 7.5	0 ± 0
(n=4)	(92%) a	(0.9%) f	(0.5%) i	(1.6%) k	(0.1%) m	(0%) o
Autumn	5281.3 ± 615.8	1595.7 ± 679.3	31.3 ± 31.3	15.7 ± 11.1	249 ± 78	27 ± 20.8
(n=3)	(73%) b	(22%) f	(0.4%) i	(0.2%) k	(3.5%) m	(0.4%) o
Winter	7081 ± 119	0 ± 0	0 ± 0	0 ± 0	119 ± 119	0 ± 0
(n=3)	(98.4%) a	(0%) f	(0%) i	(0%) k	(1.6%) m	(0%) o
Spring	2955 ± 974	3625 ± 833	34 ± 12.5	416 ± 187	142.75 ± 49.7	27 ± 16.3
(n=4)	(41%) c	(50.3%)g	(0.5%) i	(5.8%) k	(2%) m	(0.4%) o
Ghrelin						
Summer	4818 ± 4818	1726 ± 1726	153.5 ± 153.5	386.25 ± 386.2	589 ± 89	26.5 ±26.5
(n=4)	(66.9%) de	(24%) h	(2.1%) j	(5.4%) l	(1.2%) n	(0.4%) p
Autumn	4498 ± 1064.5	1292 ± 598	228 ± 164.1	327.7 ± 316.2	845.3 ± 249.1*	9 ± 5.2
(n=3)	(62.5%) de	(17.9%) h	(3.2%) j	(4.5%) l	(11.7%) n	(0.1%) p
Winter	6325.7 ± 94*	161 ± 161	14.7 ± 7.3*	0 ± 0	698.7 ± 179*	0 ± 0
(n=3)	(87.9%) d	(2.2%) h	(0.2%) j	(0%) l	(9.7%) n	(0%) p
Spring	2613.25 ± 890	3593.5 ± 748*	230.5 ± 88.3*	413.25 ± 150	301.5 ± 109.2	48 ± 19.8
(n=4)	(36.3%) e	(49.9%) h	(3.2%) j	(5.7%) l	(4.2%) n	(0.7%) p

N.S. Activity = non-specific activity. Mean time (in seconds) per group ± SEM (percent of total time in parentheses calculated out of 7200 seconds (2 hours) post injection). * = ghrelin different from saline (same season, same activity). Numbers with differing letters (eg. a,b,c) indicate significant difference between seasons within an activity and treatment type. All differences are considered significant at $p \leq 0.05$.

In summer, animals injected with ghrelin showed no significant differences in behavior from those injected with saline. In autumn, ghrelin-injected animals spent significantly more time nesting than saline controls. In winter, ghrelin-injected animals spent significantly less time inactive, and more time feeding and nesting than did the controls. In spring, ghrelin-injected animals spent significantly more time than controls in non-specific activities (generally moving around the cage) and feeding.

By season, both control and experimental animals were most active in spring (Table 2), equally active in summer and autumn, and least active in winter. All animals spent significantly more time in non-specific activity in spring than in other seasons.

Ghrelin also appeared to alter the percentage of active time that animals were engaged in specific behaviors (Figure III.B.7).

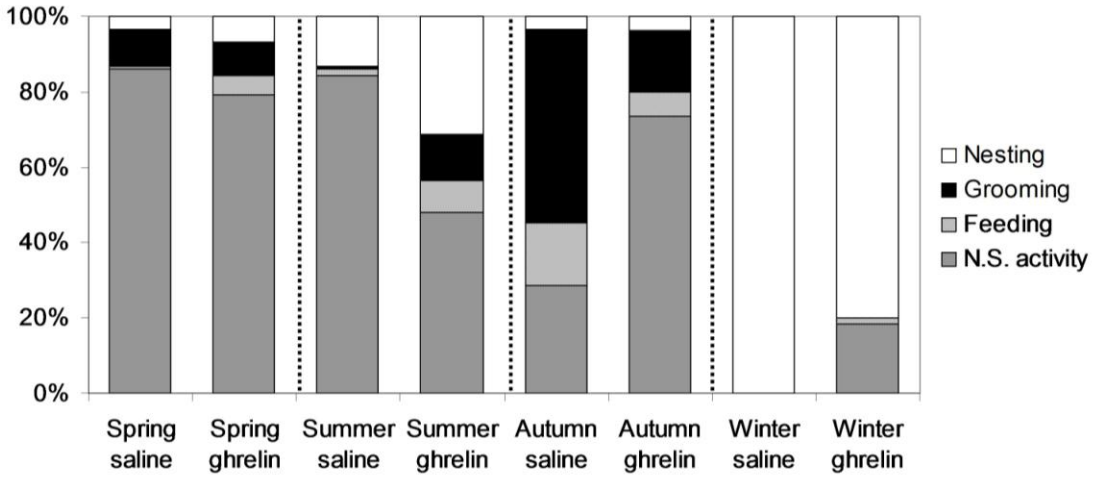

Figure III.B.7: Breakdown of percent total time active spent in specific behaviors

In spring, summer, and winter, animals spent the majority of their active time engaged in non-specific behavior (up to 85%), with considerable amounts of time spent grooming (up to 50%) and nesting (up to 30%). Time spent feeding was between 1% and 16% of total time active. In winter, the majority of time active was spent nesting (100% for saline injected animals and 80% for ghrelin-injected animals), with 18% of time spent in random activity and 2% spent feeding in the ghrelin-injected animals (Figure III.B.7).

III.B.5. *Discussion*

The orexigenic effect of ghrelin in most mammals is well documented, but this study is the first to show an effect of ghrelin on winter-acclimated golden-mantled ground squirrels. We injected ghrelin peripherally into squirrels during all four seasons

and measured changes in food intake, body mass, and activity levels for 6 hours post-injection. We observed an increase in food intake in all squirrels injected with ghrelin—animals injected with ghrelin ate 2-4 times as much as the saline controls, even during winter when animals are normally aphagic. Animals aroused to euthermia were induced to eat by injection of ghrelin, while animals injected with saline remained aphagic, and indeed attempted to return to torpor within two hours of being aroused. A similar food intake response was recently elicited in another hibernating sciurid (*Marmota flaviventris*), which showed that central infusion of the AMPK agonist 5-aminoimidazole-4-carboxamide 1 B-D-ribofuranoside (AICAR) in winter aphagic marmots caused an initiation of food intake and cessation of torpor bouts while saline-infused animals remained aphagic and re-entered torpor even when held at high Ta (Florant et al., 2010).

Animals injected with ghrelin generally had higher body masses than saline-injected controls two hours after injection, probably due to the increase in food intake. Body mass in GMGS tends to fluctuate widely throughout the day (from 2-10 grams, Florant lab unpub. data) based on animals' activity levels and timing of food intake, especially in summer when ground squirrels maintain a consistently high metabolic rate (Armitage and Shulenberger, 1972). Both saline-injected and ghrelin-injected groups of summer animals lost body mass over the two hour post-injection period, but the mass loss was attenuated by ghrelin-induced food intake.

Ghrelin-injected animals had higher serum ghrelin concentrations than those injected with saline in both winter and summer, but the ghrelin injections led to a proportionally greater increase in serum ghrelin in the winter than in summer. However,

winter ghrelin-injected animals ate far less than summer ghrelin-injected animals, so it is possible that the lower metabolic and circulation rates of the winter squirrels delayed the absorption and distribution of the injected ghrelin bolus, leading to higher circulating levels when animals were sacrificed two hours after injection. In other mammals, ghrelin stimulates food intake by crossing the blood-brain barrier and binding directly to the GHSR1 receptor in NPY/AgRP neurons, and stimulating the release of these neuropeptides (Kamegai et al., 2001; Seoane et al., 2003). Usually, circulating ghrelin concentrations are directly correlated with food intake (Cummings, 2006; Keen-Rhinehart and Bartness, 2005), but in our animals, circulating serum ghrelin appears to have a diminished effect on the food intake of aphagic winter-acclimated ground squirrels compared with normophagic animals. This is similar to effects seen recently in another seasonal rodent, the photoperiodic Siberian hamster (*Phodopus sungorus*), which decreases food intake and undergoes shallow torpor bouts when acclimated to short-day length (SD). SD acclimated hamsters showed decreased sensitivity to ghrelin when compared with long-day (LD) acclimated animals (Bradley et al., 2010).

The increase in food intake exhibited by GMGS after ghrelin injection was accompanied by an increase of the phosphorylated (active) form of AMPK in both summer and winter, as seen in previous research (Kohno et al., 2008). Summer ghrelin-injected animals had increased levels of both total and phosphorylated AMPK when compared with saline-injected controls, suggesting that in summer hibernators, ghrelin injection stimulates AMPK release, but does not increase the rate of phosphorylation. In winter ghrelin-injected animals, we saw an increase in the phosphorylated form of AMPK in the hypothalamus, but there was no difference in total levels of AMPK, which

suggests that ghrelin stimulated a larger proportion of total AMPK to be phosphorylated and activated.

In mammals, the phosphorylation of AMPK usually causes the concomitant phosphorylation and deactivation of ACC. Since ACC is a committed step in fatty acid synthesis, its deactivation effectively causes a switch from fatty acid synthesis to fatty acid oxidation. We measured total and phosphorylated levels of ACC in the hypothalami of ghrelin-injected and saline-injected animals. In summer, ghrelin-injected animals had increased levels of both phosphorylated and total ACC, as seen in previous research (Kohno et al., 2008), but we saw no difference in either total or phosphorylated ACC between groups in the winter. Since winter hibernators are typically aphagic and lipolytic, it is possible that ghrelin stimulated the phosphorylation of AMPK to cause an increase in appetite, but since animals had ceased fatty acid synthesis prior to entering hibernation, ACC was already maximally phosphorylated.

We also found a significant increase in circulating serum NEFA concentrations after peripheral ghrelin injection. Since ghrelin's primary role is to stimulate appetite, and secondarily increase fatty acid synthesis (over the long term), it seems plausible that an increase in circulating ghrelin might initially raise circulating NEFAs concurrently with increasing food intake, but eventually cause a decrease in circulating NEFAs as fatty acid synthesis was stimulated (Theander-Carrillo et al., 2006). It is also possible that due to the acute and transitory nature of the peripheral injection, and the short time after injections in which the animals were sacrificed, that the observed increase in NEFAs after ghrelin injection was due to the breakdown of the food recently consumed (some food was found in the stomachs of ghrelin-injected animals).

Activity levels of GMGS generally increased with ghrelin injections, especially random activity and feeding/food seeking behaviors. Ghrelin has been shown to increase random locomotor activity levels in rats and mice (Jazberenyi et al., 2006; Jerlhag et al., 2006), in apparent contradiction to its well-known lipogenic effects. This effect on locomotor activity may be due to ghrelin's amplifying effect on central dopamine (DA) neurons (Jerlhag et al., 2006). However, this increase in locomotor activity appears to be dose dependent—as various behaviors are stimulated by different areas of the brain (e.g. hypothalamus controls ghrelin's effect on feeding behavior; cerebral cortex controls ghrelin's effect on memory), these brain regions are also differently sensitive to ghrelin level (see Ferrini et al., 2009 for a review). It is likely that the increase in active behavior resulting from ghrelin injections in our experiment is due to the intensified appetite stimulus usually associated with injection of this peptide.

Hibernators represent a unique animal model for the study of human obesity and a 'natural knock-out' for research on controls of food intake. These animals are normally completely aphagic during their winter hibernation season, so it is significant that injection of a single peptide induced GMGS to commence food intake. Further experiments are needed to elucidate ghrelin's effect on hibernators during the aphagic hibernation season and during the summer hyperphagic season. Nonetheless, this initial study indicates that ghrelin does have an effect on hibernators, even during a period of time when they do not normally eat.

III.B.6. *References*

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Chapter IV: AMPK and ACC

IV.A. Fasting increases activity of AMPK and fatty acid oxidation in euthermic and hibernating golden-mantled ground squirrels.

IV.A.1. *Abstract*

AMP-activated protein kinase (AMPK) is a cellular energy sensor which responds to low endogenous energy stores by stimulating catabolic (ATP-producing) pathways such as fatty acid oxidation (through the inactivation of acetyl-CoA carboxylase (ACC)) and food intake. In most mammals, fasting stimulates phosphorylation of AMPK (activated, pAMPK) and ACC (deactivated, pACC), but how AMPK and ACC react to a long-term fast, such as that seen during hibernation, is unclear. We performed western blots for total and phosphorylated AMPK and ACC on tissues from a species of hibernator (golden-mantled ground squirrel, GMGS) sacrificed after a short-term fast in summer (1-5 days) and a long-term winter fast (3 months). Winter animals were sacrificed during hibernation at low tissue-temperature (torpid, Tb~5ºC) or at normal high body temperature (euthermic, Tb~37ºC). We found a general increase in pAMPK in most tissues (liver, muscle, white adipose tissue (WAT), but not hypothalamus) and pACC in all tissues after a short-term fast in summer. Response of AMPK and ACC to a long-term winter fast differed by tissue—in liver and muscle, there was no difference in total or phosphorylated AMPK or ACC between groups, but in WAT and BAT, euthermic GMGS had lower expression of pAMPK and pACC than torpid animals.

IV.A.2. *Introduction*

AMP-activated protein kinase (AMPK) is an important metabolic regulator that senses decreasing cellular energy status and responds by stimulating ATP producing pathways, such as fatty acid oxidation and glycolysis. AMPK is an intracellular heterotrimeric enzyme and is activated by phosphorylation when exposed to high AMP:ATP ratios seen during periods of low cellular energy (Hardie et al., 2006). Acetyl-CoA carboxylase (ACC) is a multi-domain enzyme that catalyzes the conversion of acetyl-CoA to malonyl-CoA, which is an important step in lipid storage. ACC is inactivated by reversible phosphorylation by AMPK. Inhibition of ACC decreases malonyl-CoA levels; since high malonyl-CoA levels are needed to inhibit carnitine palmitoyl transferase-1 (CPT1) and decrease mitochondrial oxidation of fatty acids, inhibition of ACC allows increased fatty acid oxidation.

AMPK has distinct and differing effects on peripheral and central tissues. In peripheral tissues, AMPK regulates a variety of metabolic pathways that support catabolic (ATP-producing) processes. It stimulates fatty acid oxidation in various tissues, inhibits fatty acid synthesis in liver and adipose tissue, and inhibits protein synthesis in liver and muscle (Xue & Kahn, 2006). It reacts to fasting in various ways in different peripheral tissues. In Wistar rats, short-term fasting (19-39 hours) resulted in an increase of pAMPK and pACC in WAT, but not in liver or muscle tissue (Kajita et al., 2008). Mice fasted for 6-24 hours had increased pAMPK in epididymal but not in subcutaneous WAT deposits (Sponarova et al., 2005). Long term caloric restriction in Wistar rats appeared to decrease pAMPK in the liver, but had no effect on pACC (To et al., 2007). Centrally, Sprague-Dawley rats fasted for two days had increased pAMPK in the

ventromedial area of the hypothalamus (Murphy et al., 2009); several other studies have reported similar results in other areas of the hypothalamus (for a review, see Minokoshi et al., 2004).

Hibernation is a life history strategy found in many mammalian clades during which animals voluntarily fast for 6-8 months out of the year. Mammals that hibernate (hibernators) exhibit a robust circannual cycle of fluctuating food intake. In spring and summer, hibernators are normophagic (food intake similar to that of non-hibernating rodent), but in autumn, animals become hyperphagic (eating 2-3 times as much food as during spring and summer). At the end of this hyperphagic period, hibernators decrease and eventually completely cease food intake (aphagia) and become heterothermic, dropping their body temperature (Tb) to near ambient temperature (Ta) during multi-day torpor bouts throughout the winter season. These multi-day torpor bouts are interrupted by brief periods (12-24 hours) of euthermia (interbout arousals) before returning to low tissue temperature (torpid). During torpor bouts, hibernators maintain very low cardiovascular and metabolic rates and metabolize almost entirely fat (Dark, 2005). In some climates, hibernators may remain aphagic from late August to mid May (Florant et al., unpub. data).

The effects of short-term fasting on AMPK in various tissues have been investigated extensively with conflicting results, but few have studied the effects of AMPK during the long-term fast exhibited by hibernating mammals. For example, Horman et al. (2005) found that during hibernation, AMPK was activated in white adipose tissue (WAT) in thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*, aka *Ictidomys tridecemlineatus* after Helgen et al., 2009), but not in liver, muscle, brown

adipose tissue (BAT) or brain as compared to summer euthermic ground squirrels. However, this experiment did not differentiate between the torpid and winter euthermic (interbout arousal) stages of hibernation, and measured AMPK in the brain in a very non-specific way. Information on how AMPK acts in specific areas of the brain, such as hypothalamus, where AMPK acts as a fuel sensor, is lacking. Recent experiments by Florant et al. 2010) demonstrated that central infusion of the AMPK agonist 5-aminoimidazole-4-carboxamide 1 B-D-ribofuranoside (AICAR) into the third ventricle of winter aphagic marmots caused an initiation of food intake.

In order to elucidate the effects of both long and short-term fasting on AMPK and ACC in hibernators, we compared these enzymes in tissues from a locally abundant species of hibernator (the golden-mantled ground squirrel, *Callospermophilus lateralis*) after a short-term (1-5 days) fast in the summer and during a long-term fast (3 months) in the winter while torpid and euthermic. We hypothesized that a summer fast would increase the active form of AMPK (pAMPK) and the inactivated form of ACC (pACC) in liver, muscle, WAT, and in the hypothalamus as squirrels metabolized endogenous energy stores. In winter animals, we hypothesized that euthermic squirrels would have higher total and phosphorylated levels of AMPK and ACC than torpid animals in liver, muscle, WAT, and BAT, since these enzymes are broken down fairly quickly, and protein synthesis during torpor is minimal.

IV.A.3. *Methods*

IV.A.3.1. Animals

Adult golden-mantled ground squirrels (GMGS) were live trapped in Larimer and Gunnison Counties, Colorado, and brought to CSU under an approved IACUC protocol.

Animals were kept in a temperature controlled room under natural photoperiod and with food and water available ad libitum. Food intake was measured weekly. In September, temperature in the animal room was decreased to 5ºC, and GMGS were kept in constant darkness as they began to undergo torpor.

GMGS were randomly assigned to two groups: summer-fast and winter-fast animals. Summer-fast animals were either fed ad lib (control) or fasted one, three, or five days (n=3-4 per group), then euthanized and tissues removed. Body mass was measured immediately before food was removed, and again at time of euthanasia. Briefly, animals were anesthetized with a ketamine-acepromazine-xylazine cocktail (75%:15%:5% respectively), weighed, and decapitated. Blood was centrifuged, serum removed, and stored at -80ºC until assays. Tissues were removed, flash-frozen in liquid nitrogen, and stored at -80ºC until use.

Winter-fast GMGS were allowed to undergo hibernation until January, and were then euthanized either during an interbout arousal (euthermic, Tb~37ºC, n=4) or at low tissue temperature (torpid, Tb~5ºC, n=5). All winter-fast animals had been completely aphagic since October. Euthermic animals were aroused to euthermia by physical manipulation, allowed to remain euthermic for 3 hours, and then euthanized as described above. Torpid animals had low Tb verified by skin, mouth, and blood temperature readings by thermocouple (Thermochron), and then were decapitated without anesthesia while at low tissue temperature. Tissues and blood were removed and treated as described above.

IV.A.3.2. Western blots

Tissues were homogenized in 1ml lysis buffer containing a protease inhibitor cocktail, centrifuged, and the supernatant removed. Protein concentration was determined by BCA assay, and western blots were performed. Briefly, sample proteins were separated by SDS-PAGE and transferred to nitrocellulose. 100µg of protein were added to each gel, with β-actin as a loading control. The membranes were blocked in TBS with 5% milk powder and incubated overnight on an orbital shaker at 4ºC in primary antibody (diluted 1:1000 for pAMPK, AMPK, pACC, ACC, and β-actin). Antibodies were obtained from Cell Signaling (Phospho-AMPKα (Thr172) Rabbit mAb #2531, AMPKα (23A3) Rabbit mAb #2603, Phospho-Acetyl-CoA Carboxylase (Ser79) Antibody #3661, Acetyl-CoA Carboxylase (C83B10) Rabbit mAb # 3676, β-actin Antibody # 4967). After washing in TBST, membranes were incubated at room temperature for 1 hr in HRP-conjugated secondary antibody (1:2000) and HRP-conjugated anti-biotin antibody (1:1000). After further washing in TBST, membranes were developed by chemiluminescence (Amersham ECL Plus from GE Healthcare), and imaged on a STORM. Protein expression was quantified using ImageQuant and normalized against β-actin.

IV.A.3.3. Statistics

Statistics were performed using Graph Pad Prism 5. Differences between fasting states were determined using a one-way ANOVA followed by a Bonferroni post-test for multiple comparisons, while differences between hibernating and euthermic GMGS were determined using Student's t-test coupled with an F test for homogeneity of variance. All results were considered significant at $p \leq 0.05$.

IV.A.4. *Results*

IV.A.4.1. Summer short-term fast

Animals fasted for 1, 3, and 5 days lost progressively and significantly more mass than ad-lib fed controls, which gained mass during the 5 days of the experiment (Figure IV.A.1).

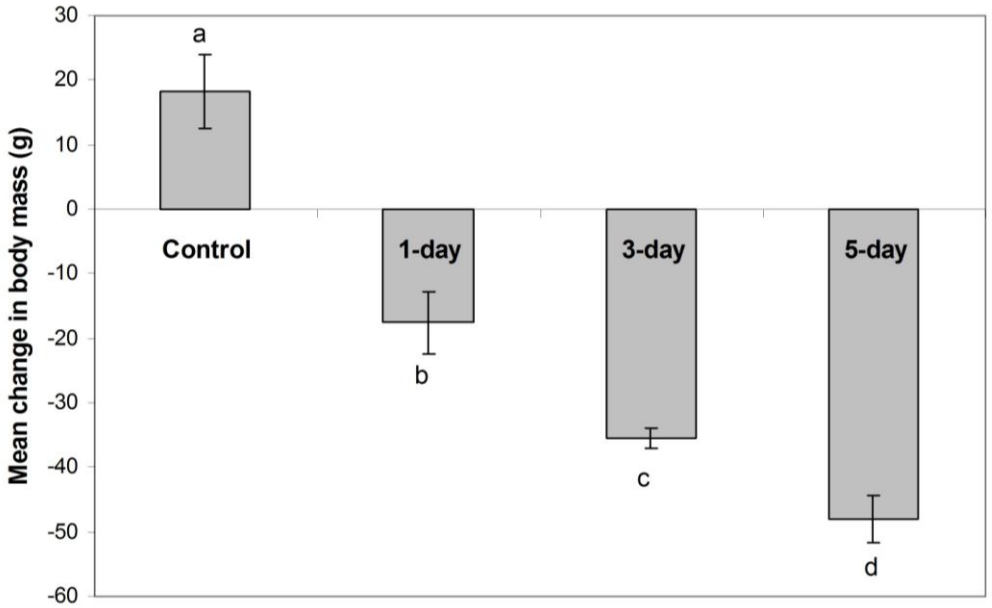

Figure IV.A.1: Change in body mass over 5 days summer fast. Each bar represents mean change in body mass ± SEM by feeding condition. Bars with different lower-case letters are statistically different ($p \leq 0.05$).

The response of AMPK and ACC to a short-term summer fast depended on phosphorylation state and tissue type (see Supplementary figure 1 for representative Western blots for summer-fast hypothalamus and muscle). In liver, fasting increased pAMPK significantly over ad-lib fed controls by day 5 of fasting, but had no significant effect on total AMPK (Figures IV.A.2.a & 2.b). The proportion of pAMPK to AMPK

increased significantly after one day of fasting; the proportion decreased after 3 and 5 days of fasting, but still remained elevated compared with controls (Figure IV.A.2.c).

Both pACC and total ACC were increased significantly after one day of fasting, but decreased to control levels by fasting days three and five (Figures IV.A.2.d &2.e). There was no difference in proportion of pACC to ACC between fasting states (Figure IV.A.2.f).

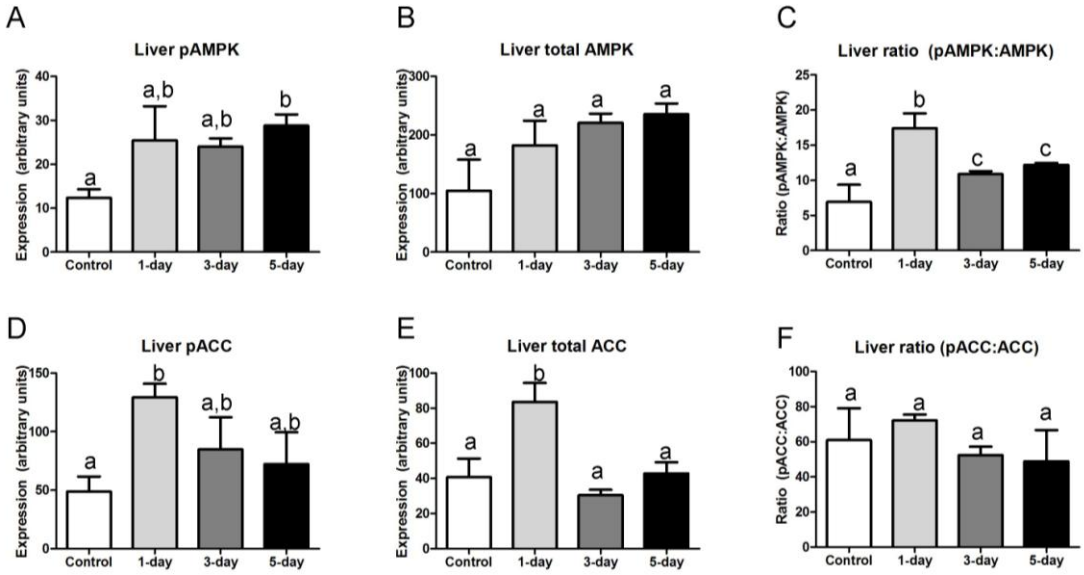

Figure IV.A.2: Total and phosphorylated AMPK and ACC in liver of animals fasted 0, 1, 3, or 5 days in July (n=3-4 per group); all bars are group means ± SEM. Bars with different lower-case letters are statistically different ($p \leq 0.05$). (A) Expression of phosphorylated AMPK; (B) Expression of total AMPK; (C) Proportion of phosphorylated to total AMPK; (D) Expression of phosphorylated ACC; (E) Expression of total ACC; (F) Proportion of phosphorylated to total ACC.

In muscle, pAMPK expression increased significantly from control levels by day five of fasting (Figure IV.A.3.a). There were no differences between fasting states in either total AMPK or the pAMPK:AMPK ratio in muscle (Figures IV.A.3.b & 3.c). Muscle pACC increased significantly with three days of fasting (Figure IV.A.3.d), but there was no difference in total ACC between fasting states (Figure IV.A.3.e). The ratio of pACC:ACC was significantly increased over control levels by day three of fasting (Figure IV.A.3.f).

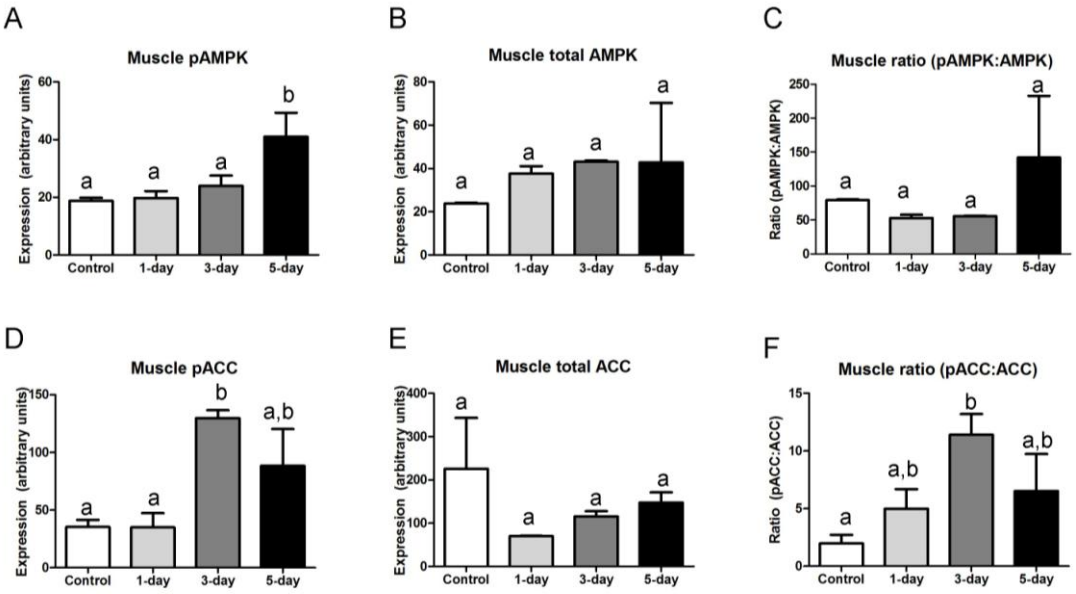

Figure IV.A.3: Total and phosphorylated AMPK and ACC in muscle of animals fasted 0, 1, 3, or 5 days in July (n=3-4 per group); all bars are group means ± SEM. Bars with different lower-case letters are statistically different ($p \leq 0.05$). (A) Expression of phosphorylated AMPK; (B) Expression of total AMPK; (C) Proportion of phosphorylated to total AMPK; (D) Expression of phosphorylated ACC; (E) Expression of total ACC; (F) Proportion of phosphorylated to total ACC.

In WAT, pAMPK increased significantly over control levels by fasting day three (Figure IV.A.4.a). There were no differences between groups in total AMPK (Figure IV.A.4.b), but the proportion of pAMPK to AMPK was significantly higher in 3-day and 5-day fasted animals than in control or 1-day fasted animals (Figure IV.A.4.c).

Expression of pACC increased significantly by day one of fasting and remained elevated in subsequent groups (Figure IV.A.4.d). Total ACC was significantly increased in 3-day fasted animals compared with controls (Figure IV.A.4.e), and the proportion of pACC to ACC was increased significantly over controls by day five of fasting (Figure IV.A.4.f).

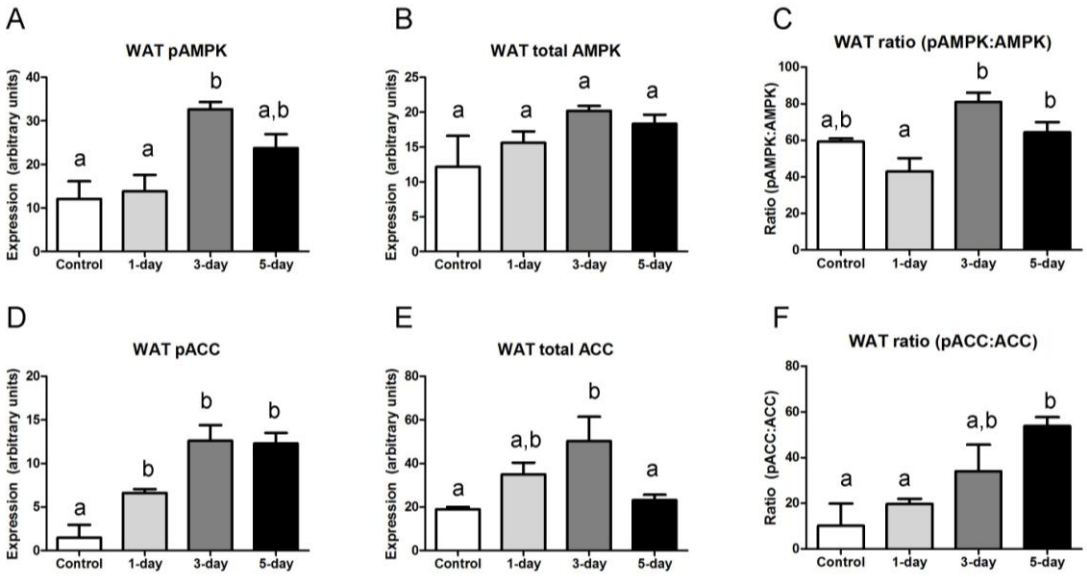

Figure IV.A.4: Total and phosphorylated AMPK and ACC in WAT of animals fasted 0, 1, 3, or 5 days in July (n=3-4 per group); all bars are group means ± SEM. Bars with different lower-case letters are statistically different ($p \leq 0.05$). (A) Expression of phosphorylated AMPK; (B) Expression of total AMPK; (C) Proportion of phosphorylated to total AMPK; (D) Expression of phosphorylated ACC; (E) Expression of total ACC; (F) Proportion of phosphorylated to total ACC.

In the hypothalamus of the brain, pAMPK increased slightly with one day of fasting ($p=0.07$), decreased from control levels after three days of fasting, and increased to control levels again by day five of fasting (Figure IV.A.5.a). There was no difference in expression of total AMPK between fasting states (Figure IV.A.5.b), but the overall proportion of pAMPK to AMPK decreased significantly from controls on day three of fasting, then increased significantly between days three and five to return to control levels (Figure IV.A.5.c). Expression of pACC increased significantly with one day of fasting

and remained elevated compared with controls for all five fasting days (Figure IV.A.5.d). There were no significant differences in total AMPK between fasting states (Figure IV.A.5.e), and the proportion of pACC to ACC increased with fasting, significantly by day five (Figure IV.A.5.f).

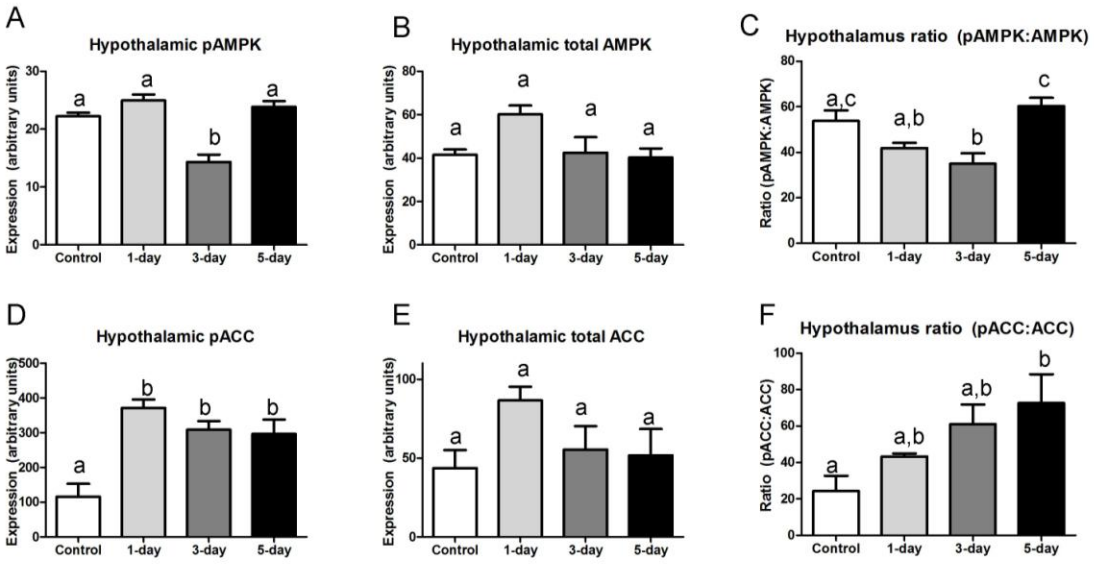

Figure IV.A.5: Total and phosphorylated AMPK and ACC in hypothalamus of animals fasted 0, 1, 3, or 5 days in July (n=3-4 per group); all bars are group means ± SEM. Bars with different lower-case letters are statistically different ($p \leq 0.05$). (A) Expression of phosphorylated AMPK; (B) Expression of total AMPK; (C) Proportion of phosphorylated to total AMPK; (D) Expression of phosphorylated ACC; (E) Expression of total ACC; (F) Proportion of phosphorylated to total ACC.

IV.A.4.2. Winter-fasted animals

Total and phosphorylated levels of AMPK and ACC were compared between two physiological states in winter-fasted animals, torpid and euthermic. In liver, there were no significant differences between states for either total or phosphorylated AMPK or ACC (Figure IV.A.6).

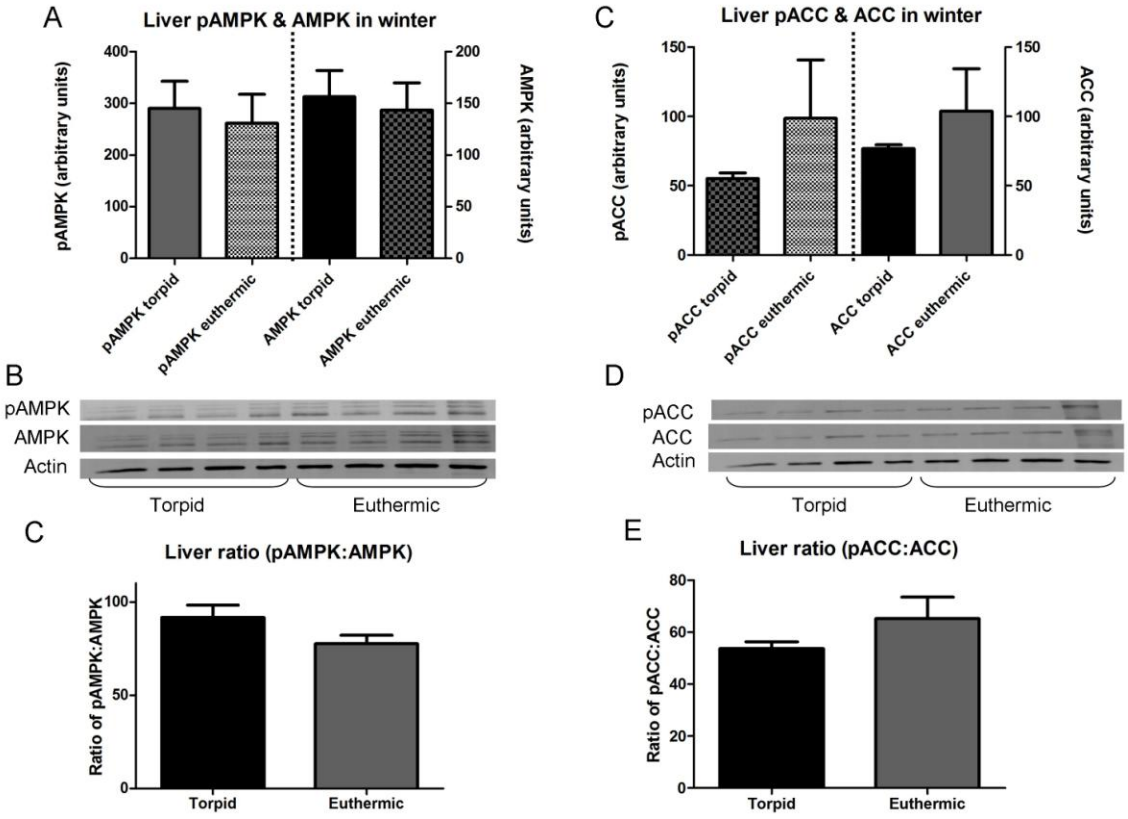

Figure IV.A.6: Total and phosphorylated AMPK and ACC in livers of animals sacrificed in January either while in torpor (torpid, n=4) or during an interbout arousal (euthermic, n=5); all bars are group means ± SEM. (A) Effect of hibernation state on hepatic AMPK (total and phosphorylated, normalized against β-actin); (B) Representative Western blots for hepatic expression of pAMPK, AMPK, and actin; (C) Ratio of phosphorylated to total AMPK (proportion of pAMPK:AMPK); (D) Effect of hibernation state on hepatic ACC (total and phosphorylated, normalized against β-actin); (B) Representative Western blots for hepatic expression of pACC, ACC, and actin; (C) Ratio of phosphorylated to total ACC (proportion of pACC:ACC).

In muscle, there were also no significant differences in either AMPK or ACC between groups, but generally euthermic animals had lower levels of enzymes than torpid animals (Figure IV.A.7). pAMPK was slightly (but not statistically) lower in euthermic animals (p=0.08), and the ratio of pAMPK:AMPK was lower in euthermic than torpid animals (p=0.07) (Figure IV.A.7.a & 7.c).

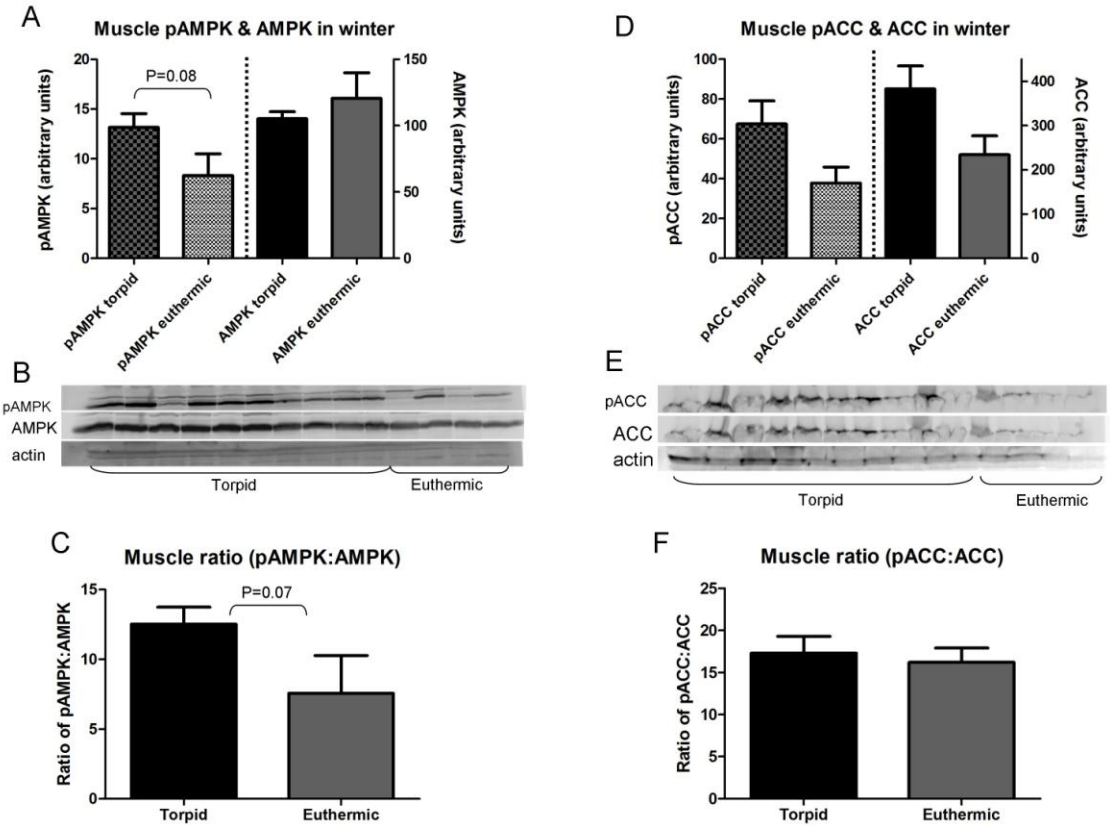

Figure IV.A.7: Total and phosphorylated AMPK and ACC in muscle of animals sacrificed in January either while in torpor (torpid, n=4) or during an interbout arousal (euthermic, n=5); all bars are group means ± SEM. (A) Effect of hibernation state on muscle AMPK (total and phosphorylated, normalized against β-actin); (B) Representative Western blots for muscle expression of pAMPK, AMPK, and actin; (C) Ratio of phosphorylated to total AMPK (proportion of pAMPK:AMPK); (D) Effect of hibernation state on muscle ACC (total and phosphorylated, normalized against β-actin); (B) Representative Western blots for muscle expression of pACC, ACC, and actin; (C) Ratio of phosphorylated to total ACC (proportion of pACC:ACC). * = euthermic significantly different from torpid ($p \leq 0.05$).

In WAT, euthermic animals had significantly ($p \leq 0.05$) lower expression of both pAMPK and total AMPK than torpid animals (Figure IV.A.8.a). There were no differences in total or phosphorylated ACC in WAT between states, or in pAMPK:AMPK or pACC:ACC ratios (Figures IV.A.8.c, 8.d, 8.f).

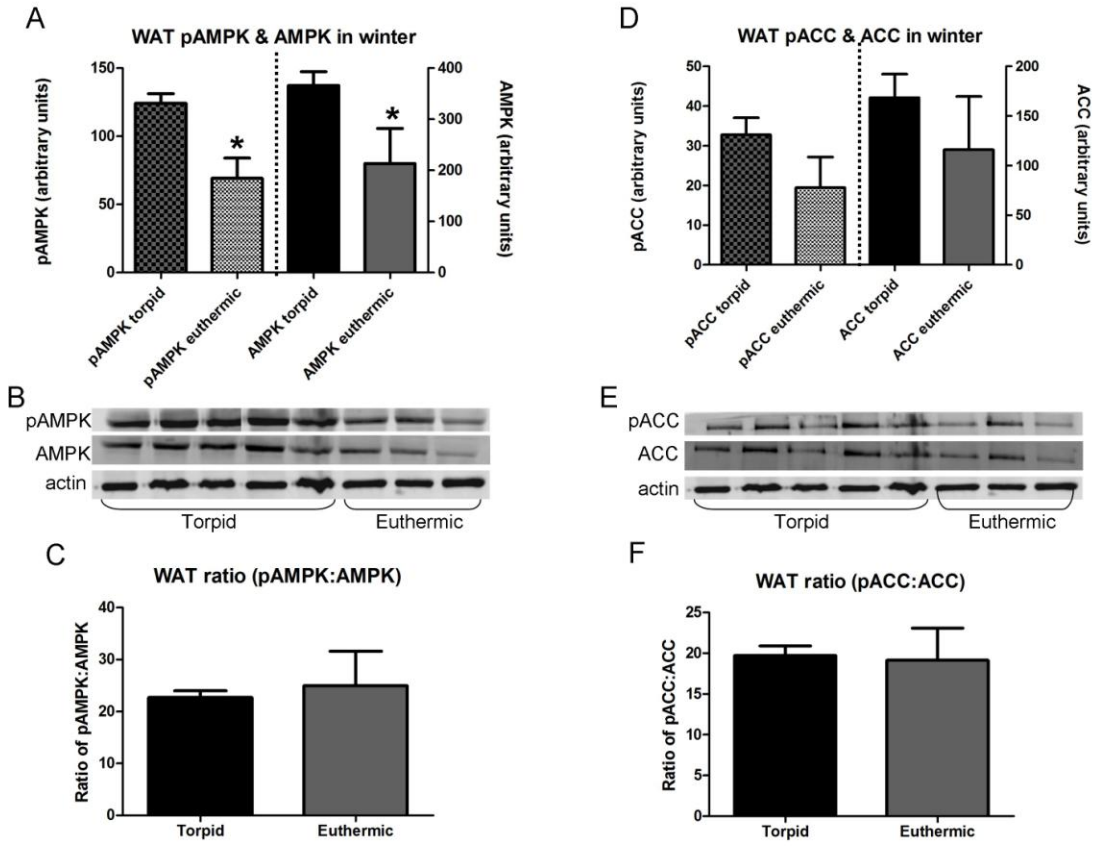

Figure IV.A.8: Total and phosphorylated AMPK and ACC in WAT of animals sacrificed in January either while in torpor (torpid, n=4) or during an interbout arousal (euthermic, n=5); all bars are group means ± SEM. (A) Effect of hibernation state on WAT AMPK (total and phosphorylated, normalized against β-actin); (B) Representative Western blots for WAT expression of pAMPK, AMPK, and actin; (C) Ratio of phosphorylated to total AMPK (proportion of pAMPK:AMPK); (D) Effect of hibernation state on WAT ACC (total and phosphorylated, normalized against β-actin); (B) Representative Western blots for WAT expression of pACC, ACC, and actin; (C) Ratio of phosphorylated to total ACC (proportion of pACC:ACC). * = euthermic significantly different from torpid ($p \leq 0.05$).

In BAT, there were no differences in total or phosphorylated AMPK between states (Figure IV.A.9.a), but both pACC and total ACC were lower in euthermic than in torpid animals (Figure IV.A.9.d). There was no difference in the proportion of pAMPK to AMPK between states (Figure IV.A.9.c), but pACC:ACC was significantly lower in euthermic than in torpid animals (Figure IV.A.9.f).

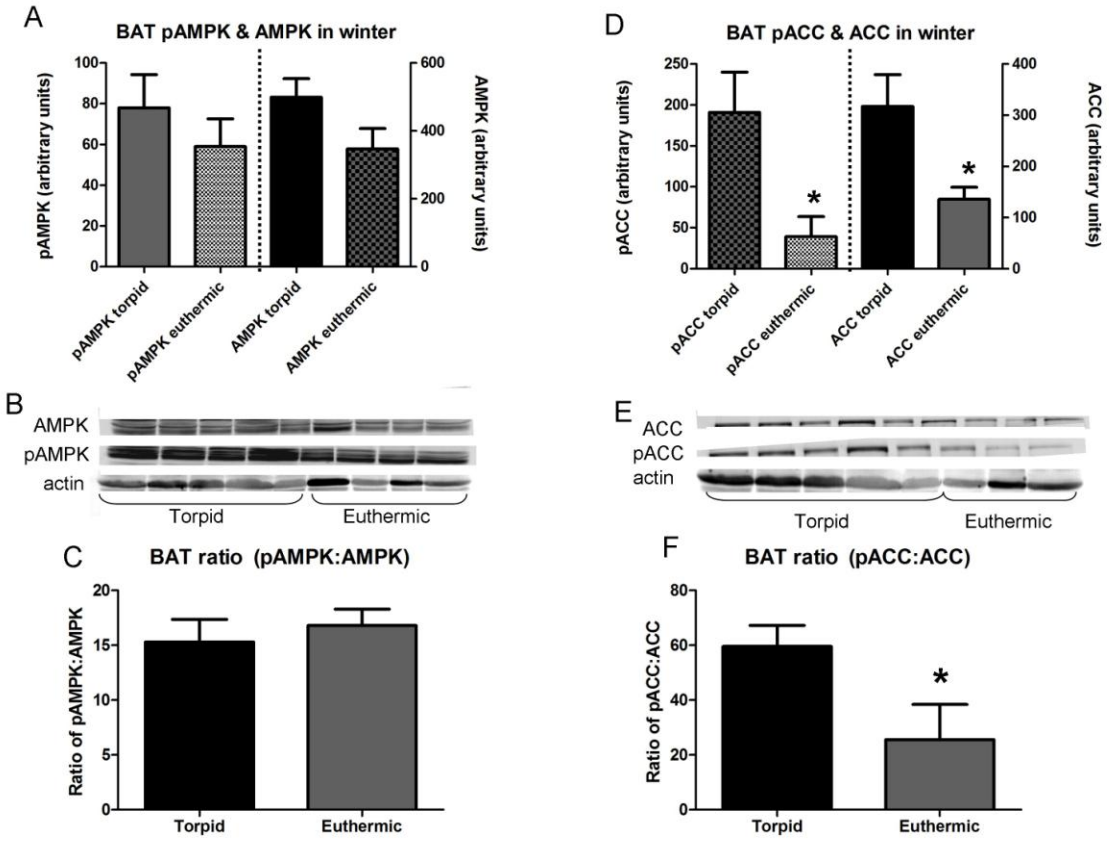

Figure IV.A.9: Total and phosphorylated AMPK and ACC in BAT of animals sacrificed in January either while in torpor (torpid, n=4) or during an interbout arousal (euthermic, n=5); all bars are group means ± SEM. (A) Effect of hibernation state on BAT AMPK (total and phosphorylated, normalized against β-actin); (B) Representative Western blots for BAT expression of pAMPK, AMPK, and actin; (C) Ratio of phosphorylated to total AMPK (proportion of pAMPK:AMPK); (D) Effect of hibernation state on BAT ACC (total and phosphorylated, normalized against β-actin); (B) Representative Western blots for BAT expression of pACC, ACC, and actin; (C) Ratio of phosphorylated to total ACC (proportion of pACC:ACC). * = euthermic significantly different from torpid ($p \leq 0.05$).

IV.A.5. *Discussion*

Hibernators require a careful balance of endogenous energy and suppressed metabolic rate in order to survive the winter season. As such, it is important for these animals to be able to sense cellular energy levels even when at low tissue temperature. Animals in deep torpor respond to changes in ambient temperature by increasing their metabolic rate (Geiser & Kenagy, 1988), and some hibernators appear to defend a 'set-point' body mass that changes throughout the year (Mrosovsky & Powley, 1977). One sensor of endogenous energy seems to be AMPK; this enzyme is important in food intake regulation and energy balance in most mammals, but has been little studied in hibernators. We compared total and phosphorylated levels of AMPK (as a cellular energy sensor) and ACC (as a downstream controller of fatty acid metabolism) in GMGS that had been fed or fasted in the summer, and in torpid and euthermic winter GMGS.

We found that summer GMGS reacted to fasting in a similar fashion to non-hibernating mammals, generally increasing the phosphorylated (active) form of AMPK with fasting, with a concurrent increase in the inactive form of ACC (pACC) in liver, muscle, and WAT. These increases in the phosphorylated forms of AMPK and ACC were usually not associated with increases in the total expression of these enzymes, effectively resulting in increased percentage of phosphorylation of both enzymes with fasting in most tissues. However, although pACC increased with fasting in the hypothalamus as expected with the increase in fatty acid oxidation that accompanies fasting, there was no associated increase in pAMPK. pAMPK increased slightly ($p=0.07$) on the first day of fasting, then decreased significantly from control levels on the third day of fasting before increasing again by day five of fasting. In previous research, mice

fasted for two days had increased expression of pAMPK in the ventromedial hypothalamus (VMH) and in various hypothalamic areas (Murphy et al., 2009; Minokoshi et al., 2004). It is possible that the brains of hibernators are protected against low energy levels even during summer, and that the increase observed in pAMPK between fasting days three and five was a delayed response to decreased endogenous energy stores. Since AMPK responds to an increase in the endogenous AMP:ATP, it is possible that enough ATP was available in the brain of fasting GMGS that the ratio did not change until after five days of fasting. To clarify this, more research is necessary on the effect of fasting on summer-acclimated hibernators, including a test to assess endogenous energy levels in the hypothalamus during a short-term summer fast.

Studies examining differences in enzyme activation in winter-acclimated hibernator tissues have generally shown enzymes to be down-regulated in torpid compared with euthermic tissues. In hearts taken from winter euthermic and summer non-hibernating Richardson's ground squirrel (*Urocitellus richardsonii*), ACC activity was decreased in the hearts of winter animals compared with summer-acclimated squirrels at both 37ºC and 5ºC. This decrease in activity was associated with a decrease in total ACC expression in winter animals, but was not associated with a change in AMPK activity (Belke et al., 1998). Other enzymes, such as skeletal muscle hexokinase (HK), creatine kinase (CK), and protein kinase C (PKC) have lower activity in torpid than euthermic hibernators, but in the case of HK, this activity is elevated by addition of AMPK (Abnous & Storey, 2001; Abnous & Storey, 2008; Mehrani & Storey, 1997). In the thirteen-lined ground squirrel (*Ictidomys tridecemlineatus)*, pAMPK expression was lower in livers of winter than summer animals, but increased in WAT in winter animals

compared with summer animals. pACC expression was elevated in the BAT of winter animals compared with summer animals, but showed no differences in other tissues (Horman et al., 2005). We did not directly compare summer-euthermic and winter-euthermic animals as seen in Horman et al. (2005), but compared state changes within seasons. Contrary to our hypothesis and to previous research, we found that expression of pAMPK and pACC was generally lower in euthermic than in torpid GMGS. There was no significant difference between total or phosphorylated forms of AMPK or ACC in liver or muscle, but both total and pAMPK were significantly decreased in WAT of euthermic GMGS compared with torpid animals, while pACC and total ACC decreased slightly. In BAT, there was a slight decrease in both pAMPK and total AMPK in euthermic animals, but the difference was significant for both total and pACC in this tissue. Although AMPK is the main cause of phosphorylation and deactivation of ACC, other enzymes have been implicated as well, including protein kinase A (PKA) (Winder et al., 1997). Both WAT and BAT are utilized heavily in the process of arousal to euthermia from torpor. It is possible that pAMPK was elevated during torpor in response to low available energy levels, and decreased when more energy became available during the interbout arousal. Alternatively, it is possible that both AMPK and ACC were maximally phosphorylated during the process of arousal to induce the massive use of fatty acids required to rewarm the animal's body from 5ºC to 36ºC over a 60 minute period, and this process of arousal expediated the breakdown of both enzyme forms. Hibernators re-build protein and enzyme stores during the euthermic portion of the interbout arousal (Epperson et al., 2010), so it is possible that at the time we chose to sacrifice the animals (two hours after arousal), the snapshot was one of low energy and

protein stores before they had time to rebuild. In order to clarify this, more research should be performed on enzyme and protein changes during each stage of the torpor bout: during entry into torpor as animals are dropping metabolic rates and Tb, in early torpor (1-2 days after reaching minimum Tb), in late torpor (after animals have been at low Tb for several days or weeks), during arousal (as animals are increasing Tb), and several hours after arousal (animals have been completely euthermic for more than six hours). Each of these stages is physiologically distinct, and may exhibit very different available energy levels, hormones, and enzyme profiles in various tissues as the body is submitted to the stresses of defending a great range of Tb.

Hibernation is a time of extreme phenotypic and physiological plasticity, and as such represents an intriguing opportunity to study animals under a wide variety of conditions and energy levels. As a sensor of endogenous energy levels, AMPK may be important in hibernators at all stages of their circannual cycle.

IV.A.6. *References*

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Chapter V: Conclusions and future directions

V.1. *Conclusions*

Hibernators have distinct physiological seasons that follow environmental seasons. During spring and summer, hibernators are normophagic and homeothermic. In autumn, hibernators become hyperphagic, and then become aphagic and heterothermic in winter. Each of these physiological states is associated with changes in concentrations of circulating hormones, expression of enzymes in various tissues, and endogenous energy stores.

In spring (March-May), hibernators are homeothermic and normophagic. In the field, adult male GMGS arouse from hibernation prior to females, eat sparingly from a stored food cache, and set up and defend a territory. Adult females and all juveniles arouse 1-4 weeks later than adult males (Kenagy et al., 1989, Florant et al., unpub. data). Adult females are impregnated within days of emergence from hibernacula, and then resume food intake. In the lab, animals are not allowed to reproduce, and as such the usual pattern of energy expenditure is attenuated. Males still arouse from hibernation and commence food intake earlier than females (Florant et al., unpub. data), but because neither undergo the energetic costs associated with reproduction, differences in circulating metabolites between sexes are minimal.

In spring lab animals, we found that circulating leptin concentrations were low compared with other seasons, correlating with decreased levels of WAT. Circulating ghrelin was also low compared with summer and autumn concentrations, suggesting that

ghrelin may remain suppressed from the winter aphagic state until the hibernator's digestive system is completely restored to its fully functional summer state from its winter shrunken state. Peripheral injection of ghrelin in spring lab animals increased circulating serum ghrelin as well as food intake, body mass, and average locomotor activity.

In the field, female GMGS have slightly higher concentrations of circulating ghrelin than males in spring (Supplementary figure 2). In the same animals, NEFAs were slightly lower in spring than during the summer (Supplementary figure 3), with a significant difference between sexes (males had lower circulating NEFAs than did females) (Supplementary figure 4).

In summer (May-August), hibernators are homeothermic and normothermic. We found that a short term fast in summer increased phosphorylation of AMPK and ACC in WAT, muscle, and liver, indicating low endogenous energy stores and fatty acid oxidation in these tissues. Peripheral injections of ghrelin increased circulating ghrelin concentrations, food intake, body mass, and average locomotor activity when compared with saline-injected controls. Injection of ghrelin also was correlated with increased expression of total and phosphorylated AMPK and ACC, indicating that the stimulation of food intake by ghrelin may be mediated by the activation of AMPK. Concentrations of circulating leptin increase as stores of WAT increase, and circulating ghrelin concentrations are increased from spring levels.

In the field, females maintain a higher ghrelin concentration than males during summer, when females are giving birth to young and lactating (Supplementary figure 2). Circulating NEFAs in field animals increase slightly in summer from spring, but the sex

difference trend in NEFAs is opposite from spring, with males exhibiting higher NEFA concentrations than females. Since NEFAs and ghrelin provide a snapshot of endogenous energy stores and requirements, it is possible that the sex difference seen in these factors in field GMGS is due to the energetic requirements of lactation placed upon females. With higher energy expenditure and lower available energy stores than males (reflected in low NEFA concentrations), females are likely to require increased food intake (which is physiologically stimulated through increased ghrelin levels) to support these energy supply needs. This hypothesis is supported by data that suggests that lab-kept animals (which are not allowed to reproduce) have no differences in circulating NEFA or ghrelin concentrations. Lab-kept animals also had higher circulating NEFA concentrations than field animals, reflecting the higher endogenous energy state of lab animals allowed *ad libitum* access to food with little opportunity for energy expenditure (Supplementary figure 5).

In autumn (August-October), hibernators are hyperphagic for the first part of the season (late July through August), before gradually decreasing food intake to zero in preparation for hibernation. Animals decrease metabolic costs in combination with increasing food intake to support dramatic lipogenesis. Ghrelin circulates at high concentrations during the hyperphagic period, increased over spring and summer concentrations (Supplementary figure 6). During this season, and presumably other seasons when hibernators are homeothermic, circulating ghrelin concentrations fluctuate throughout the day, with lower levels correlating with times of increased food intake, and high concentrations correlating with times of decreased food intake (dark hours). During autumn, fat mass increases dramatically, but circulating leptin seems to lag behind WAT

production in lab GMGS fed *ad libitum*, possibly an adaptive strategy to allow maximum storage of WAT before the winter aphagic period. Caloric restriction during this time keeps fat mass and circulating leptin concentrations low, but serum ghrelin concentrations are slightly increased over control levels (Supplementary figure 7). Near the end of the hyperphagic period, leptin levels increase dramatically, possibly providing an orexigenic signal to slow and eventually cease food intake.

In winter (October-March), animals are aphagic and heterothermic, dropping their Tb to near Ta for extended periods of time. These torpor bouts are broken by periodic interbout arousals (IBA) to euthermia, during which animals return to high Tb and resume normal physiological function for 12-24 hours. During the winter, ghrelin circulates at lower concentrations than at other times of the year (Supplementary figure 6), possibly due to the decreased size of the hibernating digestive system—with fewer ghrelin-producing cells available, less ghrelin should be circulating in the blood. Circulating ghrelin concentrations are higher during interbout arousals than during torpor at low Tb. Since hibernators spend most of an IBA undergoing sleep (Daan et al., 1991), it may be that ghrelin is exhibiting its stimulatory effect on slow-wave sleep while remaining below the physiological threshold at which ghrelin stimulates food intake. Artificial stimulation of circulating ghrelin concentrations by peripheral ghrelin injection stimulates food intake during this aphagic period. This stimulation of food intake is accompanied by an increase of the active (phosphorylated) form of AMPK, but no change in ACC, possibly due to a relative insensitivity to ACC in the hypothalamus of winter-acclimated hibernators. It is also possible that ACC is maximally phosphorylated in the hypothalamus during this time, so it is unable to react to changes in AMPK. Since

animals are typically aphagic during this season, a switch to fatty acid synthesis instead of oxidation would be detrimental, so a mechanism that in effect prevents fatty acid synthesis from taking place while animals are at low tissue temperature would be advantageous. This possibility should be investigated by comparing activity of ACC and AMPK, as well as levels of fatty acid oxidation in the hypothalami of torpid and euthermic winter-acclimated hibernators. In other tissues, however, this season-wide maximum phosphorylation of ACC does not appear to be the case—in the WAT, BAT, and muscle of winter-acclimated hibernators, pACC differs between torpid and euthermic states, and is lower in euthermic animals than torpid animals. This may suggest that ACC is maximally phosphorylated (inactive) during torpor, when animals metabolize almost entirely fat, but is allowed to be at least partially active during euthermic interbout arousals. This supports the hypothesis of Martin & Epperson (2008) that hibernation is a "two-switch" system in which gene expression (and by association, protein expression) is changed significantly between summer and winter states in order to allow animals to undergo torpor, but gene expression also changes within the winter phenotype, allowing animals to move between metabolic suppression (torpor) and normal metabolic function (euthermia).

V.2. *Future directions*

In order to clarify ghrelin's effect on downstream metabolic factors, ghrelin and saline injections should be repeated in spring, summer, autumn, winter without food available to avoid the confounding variable of digested food altering endogenous energy

levels. This would allow us to examine ghrelin's effect on AMPK and ACC in various tissues, and in circulating leptin and NEFAs.

Stomachs of hibernators under different conditions should be stained and histologically examined for changes in number and location of ghrelin-producing cells. Digestive systems of winter aphagic hibernators are typically atrophied, so it would be interesting to determine if fewer ghrelin-producing cells are active during this time. Little is known about how ghrelin-producing cells react to fasting, but one study showed an increase in ghrelin-reactive cells in the stomachs of rats during a 7-day fast; the number of ghrelin-immunoreactive cells decreased after re-feeding (Sonmez & Ozan, 2007).

Manipulation of endogenous leptin concentrations by peripheral leptin injections during all four seasons would allow us to determine if high circulating leptin can shut off food intake. This would allow us to examine the effects of injected leptin on circulating ghrelin, and gauge the strength of leptin as a satiety signal at various points during a hibernator's circannual cycle of body mass and food intake. This would also allow us to determine if hibernators are leptin-resistant at certain points during their food intake cycle, such as during the autumnal hyperphagic period.

In order to clarify physiological changes in hibernators during all phases of a torpor bout, hibernators should be sacrificed at all five stages of a torpor bout: 1) entry into torpor (animal is reducing Tb (Tb~18ºC) and metabolic rate); 2) early torpor (animal has been at low tissue temperature (Tb≤10ºC) for two days); 3) late torpor (animals has been at low tissue temperature (Tb≤10ºC) for at least fourteen days); 4) arousal (animal is actively increasing Tb (Tb~18ºC) and metabolic rate); 5) interbout euthermia (animal has

been at euthermic Tb (≥36ºC) for at least eight hours). With tissues, blood, and brains collected from animals at all stages of a torpor bout, we can more accurately track changes in circulating ghrelin, leptin, and NEFAs, tissue activation of AMPK and ACC, production of ghrelin by the stomach, levels of fatty acid oxidation, and changes in activity of various neurons in different brain regions using immunohistochemistry to compare protein changes or in situ hybridization to compare changes in RNA.

Hypothalamic cells from animals at each of the torpor stages should be treated with AICAR (an AMPK agonist) in order to directly determine how AMPK activation affects activation of ACC and of orexigenic and anorexigenic neuropeptides such as NPY, AgRP, and POMC. The lysates from the AICAR-treated hypothalamic cells will be submitted to western blot and probed for the above named peptides.

These and many other experiments will be necessary to more fully understand the controls of the food intake pathway in hibernators, but examination of the seasonal changes in the hormones ghrelin and leptin and the enzymes AMPK and ACC have yielded intriguing results that may shed some light on the mechanisms underlying the adaptive life-history strategy of hibernation.

The above data seems to indicate that hormones change in a manner consistent with the physiology of hibernators, but these changes are different than those seen in non-hibernating rodents. Ground squirrels overeat and become obese, but remain healthy. Their circulating hormones and neuropeptides change, but in some cases the hormones seem to have a more reactive than a regulatory role. Leptin may remain depressed while white adipose tissue is increasing, possibly by being inhibited due to the high levels of ghrelin circulating during the hyperphagic period. Ghrelin remains depressed during

winter (when GMGS fast as long as 8 months), possibly due to the shrinkage of the digestive system. These results give us a better understanding of the hormonal changes in hibernators, but our ultimate goal is to understand the physiological mechanisms underlying these hormonal changes.

V.3. *References*

Daan, S., Barnes, B.M., Strijkstra, A.M., 1991. Warming up for sleep? Ground squirrels during arousals from hibernation. Neurosci Lett 128: 265-268.

Kenagy, G.J., Sharbaugh, S.M., Nagy, K.A., 1989. Annual cycle of energy and time expenditure in a golden-mantled ground squirrel population. Oecologia 78: 269-282.

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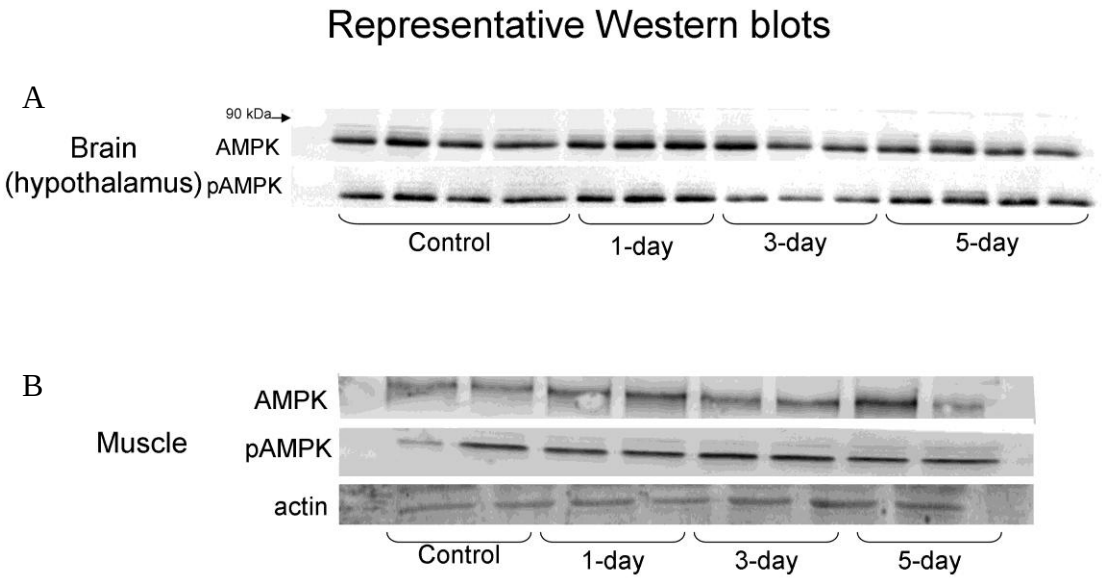
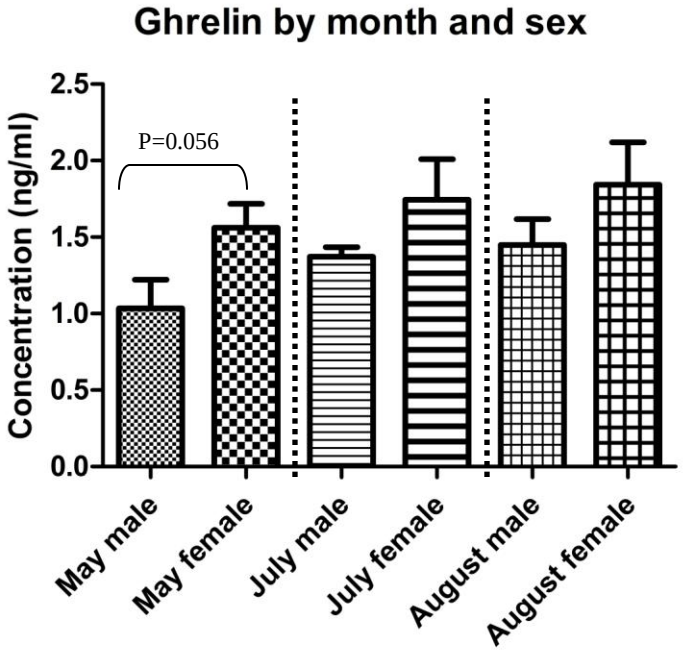
Sonmez, M. F., Ozan, E., 2007. Determination of ghrelin immunoreactivity in the rat stomach after fasting and refeeding. Acta Histochemica 109: 193–199.

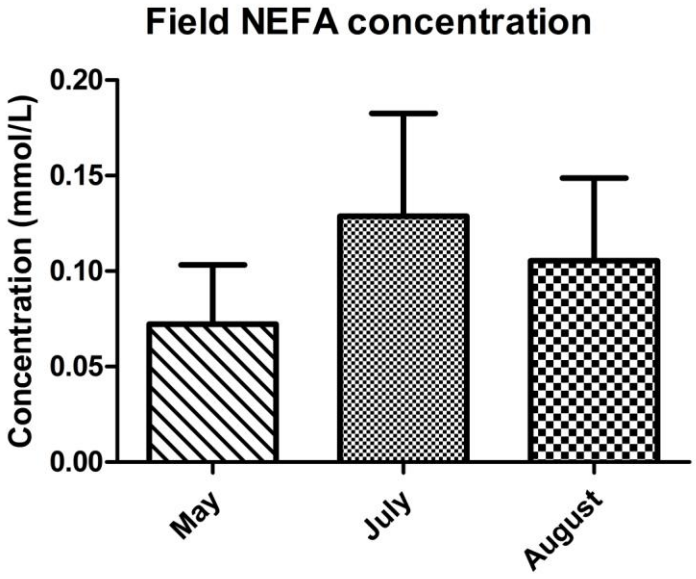
Chapter VI: Supplementary figures



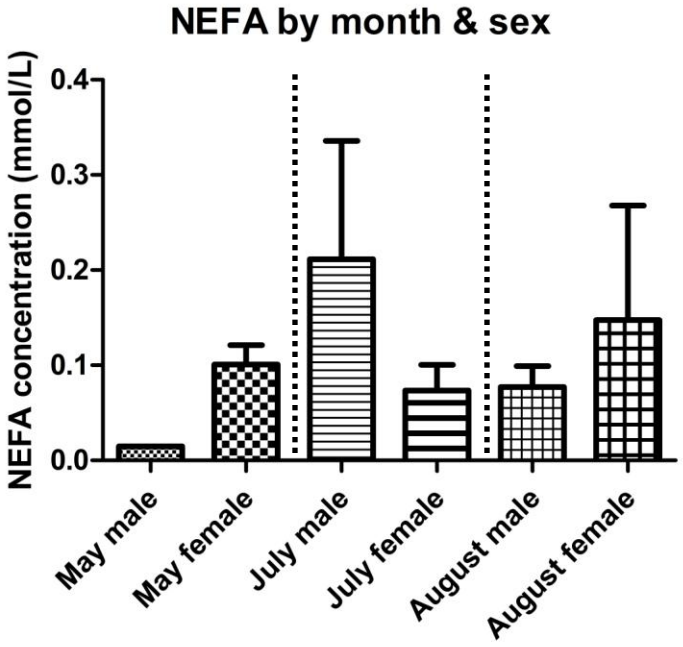
Supplementary figure 1: Representative Western blots for hypothalamus and muscle for summer fasted animals.
(A) total AMPK and pAMPK in hypothalamus; control n=4, 1-day fast n=3, 3-day fast n=3, 5-day fast n=4
(B) total AMPK, pAMPK, and actin in muscle; n=2 per group



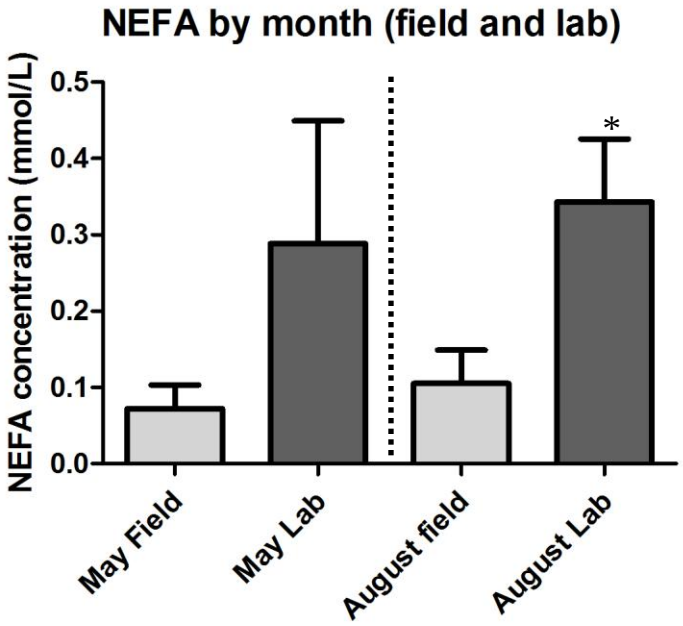
Supplementary figure 2: Differences in concentrations of circulating ghrelin from field squirrels in May, July, and August (N=4-6 per month, separated by sex)



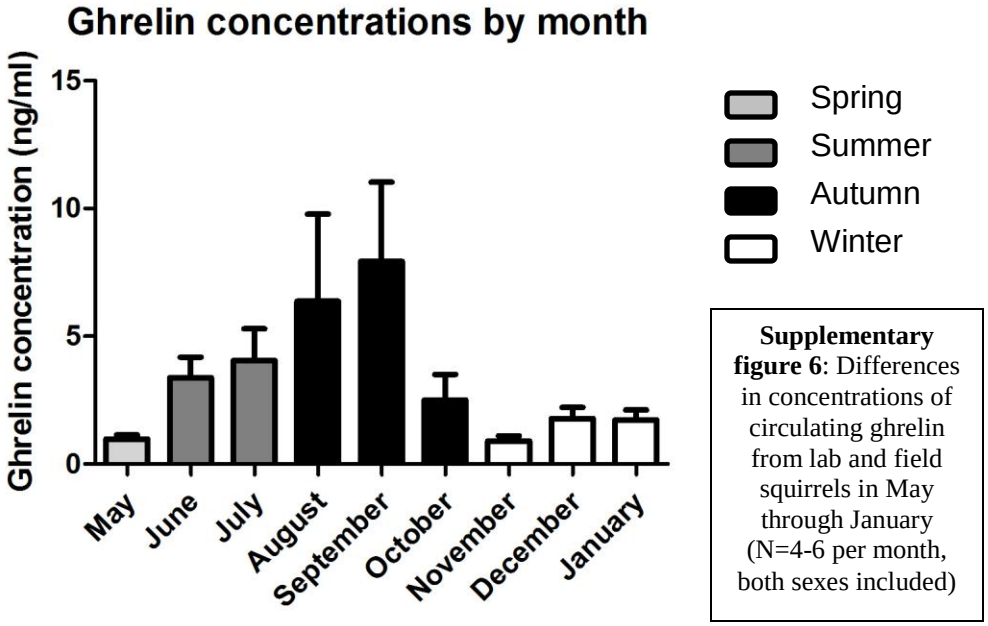
Supplementary figure 3: Concentrations of circulating NEFAs from field squirrels in May, July, and August (N=5-10 per month, both males and females)



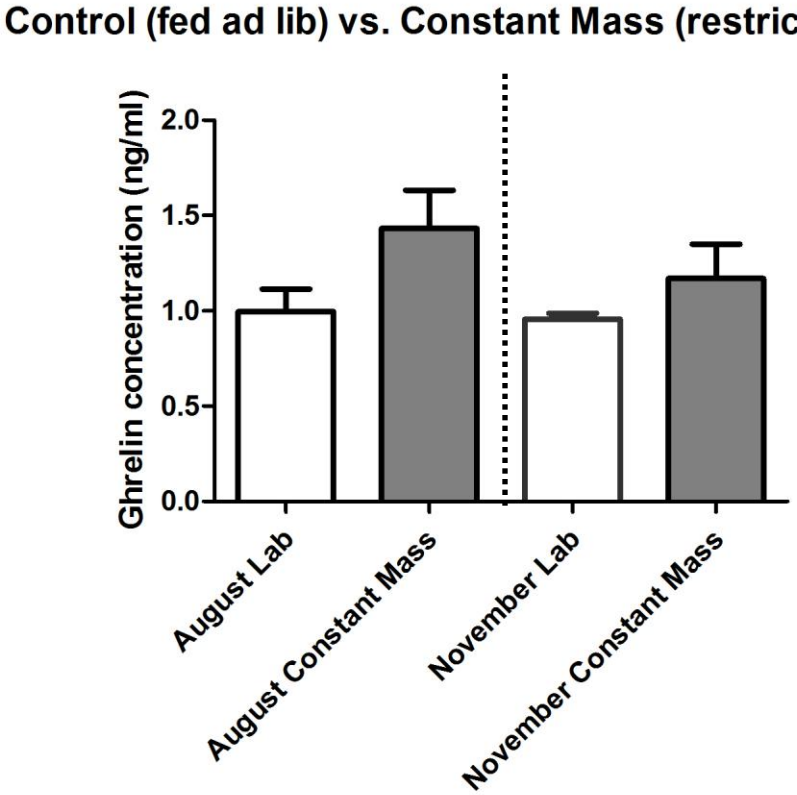
Supplementary figure 4: Differences in concentrations of circulating NEFAs from field squirrels in May, July, and August (N=5-10 per month, separated by sex)



Supplementary figure 5: Differences in concentrations of circulating NEFAs from lab and field squirrels in May and August (N=5-10 per month, both sexes included); *= lab significantly different from field in August ($p \leq 0.05$)



Supplementary figure 6: Differences in concentrations of circulating ghrelin from lab and field squirrels in May through January (N=4-6 per month, both sexes included)



Supplementary figure 7: Differences in concentrations of circulating ghrelin from control and constant mass (caloric restriction) GMGS in August and November (N=5 per group, both sexes included)