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To the Graduate Council:

I am submitting herewith a thesis written by Elizabeth Marie Hill entitled "Seasonal Changes in White Adipose Tissue in American Black Bears (Ursus americanus)." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Brynn H. Voy, Edward C. Ramsay, Major Professor

We have read this thesis and recommend its acceptance:

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(Original signatures are on file with official student records.)

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Seasonal Changes in White Adipose Tissue in American Black Bears (*Ursus americanus*)

A Thesis Presented for the

Master of Science Degree

The University of Tennessee, Knoxville

Elizabeth Marie Hill

May 2013

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DEDICATION

For Lily and Toby

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ABSTRACT

American black bears have an intricate seasonal physiology, eating an entire year's worth of food in 7-9 months, and then losing that weight during hibernation with almost no activity. The black bear thus represents a novel model in which to study seasonal regulation of food intake and metabolism. What controls the seasonal changes in fat deposition and metabolism in bears is unknown. Adipokines, such as leptin, regulate food intake and metabolism, and we hypothesized that these adipokines vary seasonally in bear adipose tissue, in a manner that correlates with fat storage. The study population consisted of wild bears from the Great Smoky Mountains National Park (GSMNP) and New Jersey and captive bears from facilities in Tennessee and North Carolina. Blood and subcutaneous fat were collected from all bears, and abdominal fat and liver samples were collected from euthanized bears. Body length and weight were measured and converted into a modified body mass index score. Circulating levels of triglycerides, non-esterified fatty acids, beta-hydroxybutyrate, leptin, and adiponectin were measured to assess lipid and glucose metabolism. A radioimmunoassay was validated for use in bears to measure serum leptin concentrations. Quantitative PCR was used to measure mRNA expression of leptin, adiponectin, pyruvate dehydrogenase kinase isoenzyme 4 (PDK4), and protein kinase, AMP-activated, alpha 1 catalytic subunit (PRKAA1) in the fat samples collected across seasons. Adipocyte size was measured as an additional index of adiposity. There were significant variations in body mass due to sampling lean bears in the GSMNP as compared to obese captive bears. PRKAA1 and adiponectin expression in subcutaneous fat were significantly greater in captive fall bears as compared to captive summer and captive winter bears.

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Circulating levels of beta-hydroxybutyrate were significantly less in captive bears as compared to wild bears. Circulating levels of leptin and leptin expression in subcutaneous fat did not change by season. Circulating levels of adiponectin were significantly higher in the fall as compared to summer and winter. Analysis of fatty acids revealed that cis-vaccenic, palmitic acid and stearic acids were prevalent in the bear. Correlation analyses identified significant relationships among adipokines, expression of metabolic genes and lipid metabolites.

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LIST OF ABBREVIATIONS

AICAR	5-aminoimidazole-4-carboxamide 1 B-D-ribofuranoside
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
β-HB	Beta-hydroxybutyrate
BP	Base pairs
BMI	Body mass index
BPM	Beats per minute
BMR	Basal metabolic rate
CRD	Completely randomized design
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
FFA	Free fatty acids
GCMS	Gas chromatography – mass spectrometry

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GSMNP	Great Smoky Mountains National Park
HINT1	Histidine triad nucleotide binding protein 1
HIT	Hibernation induction trigger
HSL	Hormone sensitive lipase
NEFA	Non-esterified fatty acids
PCR	Polymerase chain reaction
PDK4	Pyruvate dehydrogenase kinase isoenzyme 4
PPARα	Peroxisome proliferator-activated receptor alpha
PRKAA1	Protein kinase, AMP-activated, alpha 1 catalytic subunit
PUFA	Polyunsaturated fatty acids
QPCR	Quantitative polymerase chain reaction
RIA	Radioimmunoassay
RPL13	Ribosomal protein L13
RPS14	Ribosomal protein S14
RT	Reverse transcription
SQ	Subcutaneous

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TG	Triglyceride
UCP1	Uncoupling protein 1
WAT	White adipose tissue

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CHAPTER I

LITERATURE REVIEW

Physiology of hibernation

Hibernation is a dormant state that occurs when an animal is inactive, typically during the winter. The primary characteristic of hibernation is a reduced metabolic rate that allows survival during periods when food is unavailable. The evolutionary purpose of hibernation is survival. A hibernating animal would not be able to survive the cold winter expending energy to stay warm and foraging for food when there is no available food. Animals face many physiological challenges during hibernation. The primary strategy for hibernation is decreasing energy expenditure through a reduction in their metabolic rate. A hibernating animal cannot afford to expend the same amount of energy during the winter as it does during its active months. Hibernation energy is supplied by fat, which is laid down prior to hibernation.

Torpor, periods of decreased physical activity, are a component of hibernation and other strategies to survive periods of food scarcity. Hibernation, daily torpor, starvation torpor and estivation are the four types of torpor (Carey et al., 2003). Hibernation consists of prolonged periods of torpor, which can last up to six months in some species, when metabolism is drastically depressed and body temperature can drop to nearly 0 ^{o}C. Some animals, such as bats, exhibit daily torpor. They maintain normal body temperature during their active period at night, and reduce their metabolic rate during the day to conserve energy. Starvation torpor occurs when under conditions of extreme starvation, such as the laboratory mouse entering

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daily torpor when fasted (Hudson and Scott, 1979). Estivation occurs when an animal lowers its metabolic rate in response to high temperatures and low water availability.

Hibernating animals do not eat, drink, urinate or defecate. Hibernators experience a decreased heart rate, consume less oxygen, and most hibernators markedly reduce their body temperature. Energy expenditure is reduced in hibernating animals by the drastic reduction in metabolism. Hibernators are able to survive such physiological extremes and still go back to normothermic conditions without suffering any physiological consequences.

Species from many mammalian orders hibernate, but hibernation has been most thoroughly studied in rodents. Hibernating rodents, such as the marmot and ground squirrel, achieve a dramatic reduction in energy expenditure through both reduction in body temperature, to approximately 0 ^{o}C, and suppression of basal metabolic rate (BMR). Arctic ground squirrels (*Spermophilus parryii*) can lower their body temperature to -2.9 ^{o}C (Barnes, 1989). The ground squirrel has a heart rate of 3-10 beats per minute (bpm) during hibernation, compared to 200-300 bpm during normal activity periods (Lyman, 1982).

Hibernators must adapt to the circannual rhythm of fat storage, laying down fat in the fall and utilizing fat during hibernation. Those animals which do not store enough fat prior to hibernation will not survive during the winter. Yellow-bellied marmots (*Marmota flaviventris*) double their body mass during the summer, then decrease their food intake to zero before hibernation, and do not eat for almost seven months (Davis, 1976). In the laboratory, marmots

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exhibit the same behavior and do not eat from October to late March, despite food being available in their cage (Florant et al., 2010).

The fat-tailed dwarf lemur (*Cheirogaleus medius*) of Madagascar is the only primate species that hibernates. This animal differs from other hibernators in that it hibernates during the winter dry season, which is from the end of April through October. A field study of radio-collared fat-tailed dwarf lemurs revealed their body mass doubles within several weeks prior to hibernation, similar to other hibernators (Fietz and Ganzhorn, 1999).

Bats living in higher latitudes hibernate when their food, primarily insects, become scarce. The little brown bat (*Myotis lucifugus*) is a common North American bat that hibernates in large colonies in caves. These colonies huddle together for warmth. During the pre-hibernation period, the little brown bat feeds at night and normally enters torpor during the day (Kronfeld-Schor et al., 2000), increasing their body fat 32.9% (2.3 g) for males and 29.6% (2.1 g) for females during this period (Kunz et al., 1998). Little brown bats typically weigh 5-14 grams and can hibernate for over six months during the winter. Bats have a reduced heart rate of 20-40 bpm during hibernation compared to 500-900 bpm during non-hibernation (Geiser, 2001). Bats, and many other hibernators, rely on brown adipose tissue during hibernation. Brown adipose tissue provides heat essential for arousals during and at the end of hibernation (Kronfeld-Schor et al., 2000).

Some marsupials and some rodents may hibernate for very long periods and at any time of the year (Geiser, 2004). The Australian eastern pygmy-possum (*Cercartetus nanus*) is able to

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gain enormous amounts of weight quickly when food is available. This marsupial is able to store all energy as fat at any time during the year and exhibit torpor when food sources are scarce (Geiser, 2007). A laboratory study of eastern pygmy-possums found they can hibernate for a year while living off of body fat. Mean hibernation lasted for 310 ± 50 days, with the longest hibernation for a pygmy-possum lasting 367 days (Geiser, 2007). Jumping mice (*Zapus princeps*) can also estivate in spring and summer for long periods of time, with torpor lasting up to about 320 days (French, 1985).

Table 1-1 illustrates the various mammalian species that display torpor and the types of torpor they exhibit.

Table 1-1. Types of torpor utilized by mammalian taxa.

Taxa Group	Common Name	Hibernation	Daily torpor	Starvation torpor	Estivation
Marsupials	Pygmy possum & Dunnart	X	X		
Afrotheria	Tenrec	X	X	X	
Insectivora	Hedgehog	X	X		
Carnivora	Bear	X	X		
Chiroptera	Fruit bat & Little brown bat	X	X		
Primates	Fat-tailed dwarf lemur	X	X		
Rodentia	Marmot, Ground squirrel & Dormice	X	X	X	X

Redrawn based on (Letunic and Bork, 2007)

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The hibernating animal's ability to avoid becoming ketotic during hibernation has received considerable study. Ketone bodies are formed in the liver during fatty acid metabolism and excess amounts of ketones in the blood is termed ketosis. Humans and non-hibernating mammals may experience ketosis during starvation. During ketosis, an animal has a poor appetite and a decreased blood glucose concentration. The body is mobilizing fat from adipose tissue faster than the liver is able to metabolize it. During hibernation, many tissues including the brain, adapt by increasing the rate of uptake and use of ketone bodies for energy. The brain typically utilizes only glucose for energy. This allows for continuous brain function and prevents ketosis during hibernation. *In vitro* studies of hamsters and ground squirrels found that the hibernating brain metabolizes ketones (Musacchia et al., 1976). Ketoacidosis occurs when the body does not have sufficient insulin and is not able to absorb glucose. People with Type 1 diabetes are insulin deficient, which can cause the production and growth of ketones.

Since hibernating animals do not drink or urinate, water from fat metabolism fulfills their water needs (Folk et al., 1976). Hibernators, like bears, will become dehydrated and uremic during the summer, if they do not have access to water (Nelson et al., 1975). Uremia is the accumulation of nitrogenous waste products in the blood and is the result of decreased renal perfusion or renal failure. Additionally, nitrogen can be directed away from urea synthesis and toward protein synthesis pathways. The bear's ability to prevent uremia is vital to hibernation (Nelson, 1973). In a study in four adult male black bears, glycerol was found to be used as a carbon source for amino acid formation. This is another strategy to help prevent

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uremia (Ahlquist et al., 1984). Glycerol can also be a substrate for gluconeogenesis and lipogenesis during hibernation.

It is currently unknown how hibernation is induced. Hibernation induction trigger (HIT) is a substance found in the blood of hibernating animals that communicates a chemical messenger to the brain to begin hibernation. HIT is believed to be an opioid substance and there have been numerous studies to determine what exactly causes the HIT to be secreted (Horton et al., 1998).

Studies have been done to try to induce hibernation in ground squirrels and other rodents. Plasma from hibernating black bears (Ruit et al., 1987), and polar bears in various stages (winter/spring, denning/non-denning) (Bruce et al., 1990), were injected into 13-lined ground squirrels. Plasma from hibernating black bears, from non-denning winter male and female polar bears, and from a female polar bear just emerging from a winter den, all induced hibernation in the ground squirrels. Plasma from hibernating brown bears injected into Djungarian hamsters (*Phodopus sungorus*) and laboratory rats (neither species hibernates) did not stimulate metabolic suppression (Karjalainen et al., 1994). This may be due to hamsters and rats not having the specific receptors to a hibernation induction trigger substance (Hellgren, 1995) or the possibility that there is no universal or transferable hibernation induction trigger in the plasma of brown bears (Karjalainen et al., 1994).

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Ursine hibernation

Bears are large mammals in the family Ursidae and are represented by eight living species. There is considerable controversy about whether bears hibernate. Several names have been proposed for bears' winter torpor including carnivore torpor (Nelson et al., 1973), winter dormancy (Storm et al., 1988), and hibernation. Bears deviate from the rodent model of hibernation in several ways. They may wake up during the winter and roam outside the den. Additionally, their body temperature does not drop as dramatically as that of hibernating rodents. Another deviation from the more common hibernators is that female bears give birth and nurse young during hibernation. Also, in warm regions, temperate bear species may remain active year-round. Despite these variations, most individuals feel that hibernation is the best term for the bears' seasonal torpor and is the term used in this thesis (Toien et al., 2011).

Four bear species hibernate. American black bears (*Ursus americanus*), Asiatic black bears (*Selenarctos thibetanus*) and brown bears (*Ursus arctos*) undergo an annual, winter hibernation. Only pregnant female polar bears (*Ursus maritimus*) hibernate. The polar bear diet consists mainly of ringed seals, and the Arctic ice during the winter provides a platform for the bears to hunt. Male and female polar bears may dig a den during the summer and estivate during a period of food scarcity.

Four bear species do not hibernate. Giant pandas (*Ailuropoda melanoluca*) eat a diet of bamboo, and are thought to not be able to gain enough body fat in the fall to permit hibernation. Instead, giant pandas move to lower elevations during the winter, where weather

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is warmer and food is available. Sun bears (*Helarctos malayanus*) and sloth bears (*Melursus ursinus*) live in tropical climates, with year-long access to food, and do not hibernate. The spectacled bear (*Tremarctos ornatus*) lives in equatorial South America, but at high elevations, and does not hibernate.

Of the hibernating bear species, the American black bear, hereafter referred to as black bears, has received the most attention and study. Black bears experience four distinct physiological stages throughout each year (Figure 1-1). The first stage is hibernation, where bears are inactive and experience metabolic suppression. During hibernation, the bear does not eat, drink, defecate, or urinate, and relies on body fat stores to fulfill the energy demands of metabolism (Nelson, 1973). The second stage is walking hibernation, which occurs when bears leave their dens in the spring. There is minimal food and water intake for about two weeks until they begin to eat normally. This stage has been witnessed in wild (Hock, 1958) and captive black bears emerging from a hibernaculum (Nelson, 1980). The third stage is normal activity, which typically occurs from May to September when bears are active and foraging for food. The fourth stage is hyperphagia, which starts in late September until November or December when the bear begins hibernation. During this time the bear is preparing for hibernation by eating massive amounts of food. Bears are fat when entering hibernation in the late fall, but their fat is gradually expended throughout the winter so that they emerge from hibernation in the spring as lean.

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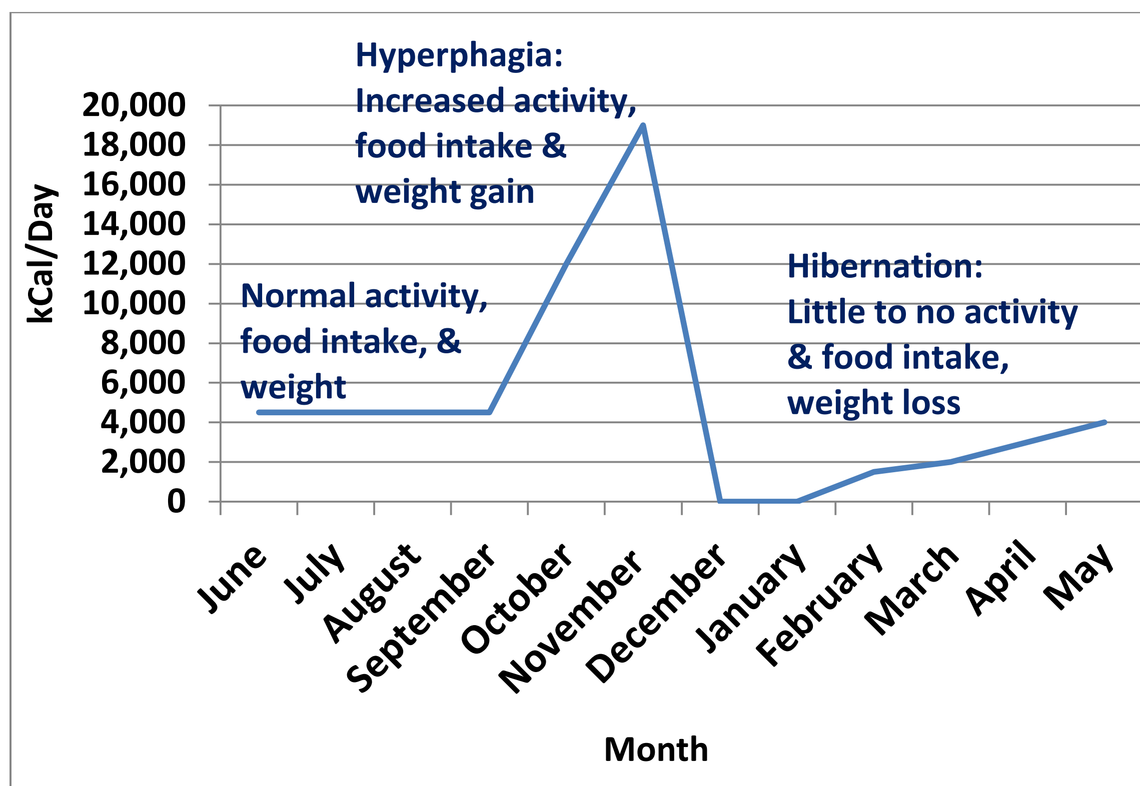

Redrawn based on (Nelson et al., 1983)

Figure 1-1. Caloric intake and major annual physiological stages for the American black bear.

Black bears share many characteristics of hibernation with other hibernating animals. Similar to other hibernators, bears experience a decrease in body temperature, heart rate and metabolic rate. While hibernating rodents dramatically decrease their body temperature around freezing, black bears undergo only slight decreases in body temperature (from a summer normal of 37-38 °C, to 30-34 °C during hibernation). The higher body temperature in bears during hibernation is mostly due to lower levels of thermal conductance in bears compared to that of rodents. Bears' thermal conductance is approximately 20% less than that of ground squirrels (Toien et al., 2011). Bears also actively suppress BMR to control energy

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expenditure (Toien et al., 2011). Similar to the small hibernators, the heart rate of black bears decreases during hibernation. Hibernating bears' heart rate can be as few as 14 bpm, increasing to 23 bpm after emergence from hibernation, and rising to 55 bpm during the summer (Toien et al., 2011). Similar hibernation related decreases in heart rate have been observed in grizzly bears (Nelson and Robbins, 2010).

Humans confined to bed rest or with an immobilized limb, have atrophy of skeletal muscle with associated loss of muscle tone and strength (Harlow et al., 2001). In ground squirrels and bats, skeletal muscle mass decreases 14-65% during hibernation (Steffen et al., 1991). In contrast, bears lose very little muscle mass during hibernation (Tinker et al., 1998). Muscle biopsies taken from denned bears during early and late winter showed no loss of skeletal muscle size or cell number (Harlow et al., 2001). Bear muscle strength does decrease during hibernation. Bear hind leg strength, determined using a non-invasive system and measured during late autumn and early spring, showed a 23% decrease after a period of inactivity for 130 days (Harlow et al., 2001). Non-hibernating, but food deprived black bears will utilize muscle mass as an energy source, similar to other warm-blooded mammals and humans , but hibernating bears do not (Nelson et al., 1975).

Similar to hibernating rodents, bear bone density is also preserved during hibernation. Bears prevent bone loss and preserve bone strength by sustaining a balance between bone formation and bone resorption (McGee-Lawrence et al., 2009). In hibernating golden-mantled ground squirrels (*Spermophilus lateralis*), bone strength is preserved after eight months of inactivity (Utz et al., 2009).

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Nelson argued that bear hibernation represents the most refined response to starvation of any mammal (Nelson, 1980). During the summer, bears are active and typically consume 5,000 to 8,000 kcal per day from May to August (Nelson et al., 1983). In the fall, preparation for hibernation requires dramatic changes in food intake. Bears begin to increase their food consumption in September and are hyperphagic through November, consuming as much as 20,000 kcal per day (Nelson et al., 1983). Wild grizzly bears have been observed feeding for 20 hours a day during their hyperphagia stage (Nelson, 1980). Bears take a minimum of two weeks to change their physiology for hibernation (Folk et al., 1972). Remarkably, captive bears, including those provided food and allowed to remain awake year-round (i.e., they do not hibernate) exhibit similar seasonal weight changes as wild bears. The Knoxville Zoo has kept a record of each bears' monthly weight, over a period of seven years. Each bear showed a distinct seasonal pattern of increasing body weight in the fall, as if in preparation for hibernation, with weights peaking in December or January. Each bear also showed weight loss through the spring, with weights troughing in early summer (Amy Flew, manuscript in preparation; Figure 1-2). This suggests that bears are physiologically programmed to alter food intake and metabolism in response to seasonal cues, independent of food availability and hibernation.

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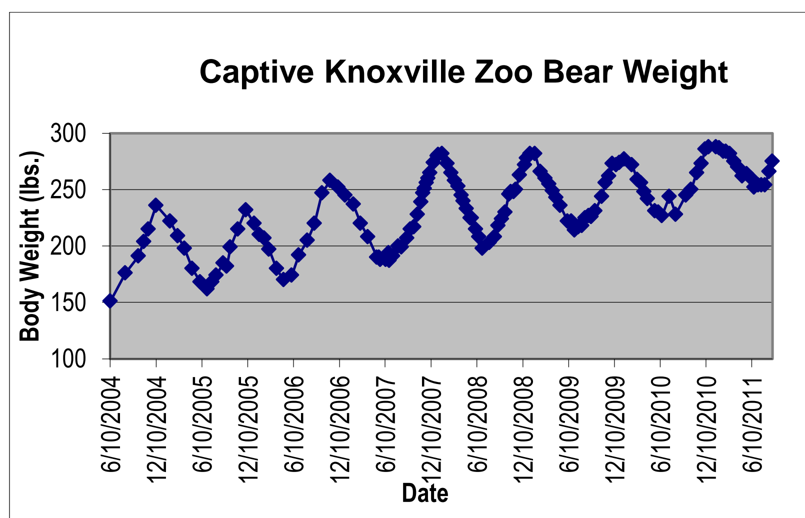

(Courtesy of A. Flew, ms in preparation)

Figure 1-2. Body weights of a captive female American black bear (*Ursus americanus*). The bear was held at the Knoxville (Tennessee) Zoo, and allowed to remain awake year-round. This bears' annual weight fluctuations were characteristic of all the Knoxville Zoo bears.

Brown adipose tissue is mainly found in human newborns and hibernating mammals, and its function is to generate body heat (Cannon and Nedergaard, 2003). The mitochondrial protein, uncoupling protein 1 (UCP1), is responsible for generating body heat (Aquila et al., 1985). Brown adipose tissue is inactive during deep hibernation and reactivates during arousal, which allows the rewarming of the hibernator to euthermia (Cannon and Nedergaard, 2003). In a hibernating and a non-hibernating black bear, it was found that brown adipose tissue is involved in cyanide-insensitive fatty acid oxidation (Davis et al., 1990). This study also found that brown adipose tissue has catalase activity and the enzymes involved in isocitrate lyase, malate synthase and the glyoxylate cycle. White adipose tissue is the product of storing excess calories, while brown adipose tissue generates heat by consuming calories. Brown adipose

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tissue is much less abundant in the bear than white adipose tissue, thus this thesis has focused on white adipose tissue.

Alterations of fat lay down and utilization are at the heart of the bears' adaptation to hibernation. While aspects of fat metabolism have been investigated, mechanisms behind its control in bears are still poorly understood. The objective of this study is to attempt to better understand the mechanisms behind the bears' dynamics of fat metabolism.

White Adipose Tissue

White adipose tissue is an energy storage depot primarily deposited in abdominal and subcutaneous fat. The principle function of adipose tissue is storing excess energy, in the form of triglycerides, and releasing the triglycerides into circulation for use by other body tissues. Triglycerides are a major component of animal fat. A triglyceride molecule consists of three fatty acids esterified to a glycerol backbone (Figure 1-3). Fats are best for fuel storage because when oxidized, one gram of fat produces 2.3 times more than oxidation of one gram of carbohydrate or protein. The fatty acids stored in adipose tissue are derived from the diet or from *de novo* synthesis in liver and adipose tissue. Triglycerides are stored in a specialized cell type, the adipocyte, which has the ability to store as well as to synthesize and release fatty acids in response to hormonal and nutritional cues. Adipocytes differentiate from a precursor, fibroblast-like cell known as preadipocytes. Preadipocytes form from a mesenchymal stem cell population that also gives rise to bone, muscle and cartilage cells (Lafontan, 2011).

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Preadipocytes differentiate into mature adipocytes under hormonal cues that induce synthesis of proteins necessary to synthesize, store, and release triglycerides. Mature adipocytes have a spherical shape and one lipid droplet surrounded by cytoplasm (Lafontan, 2011). Mature adipocytes comprise ~80% of adipose tissue mass. The remainder of adipose mass consists of preadipocytes, endothelial cells, lymphocytes and macrophages.

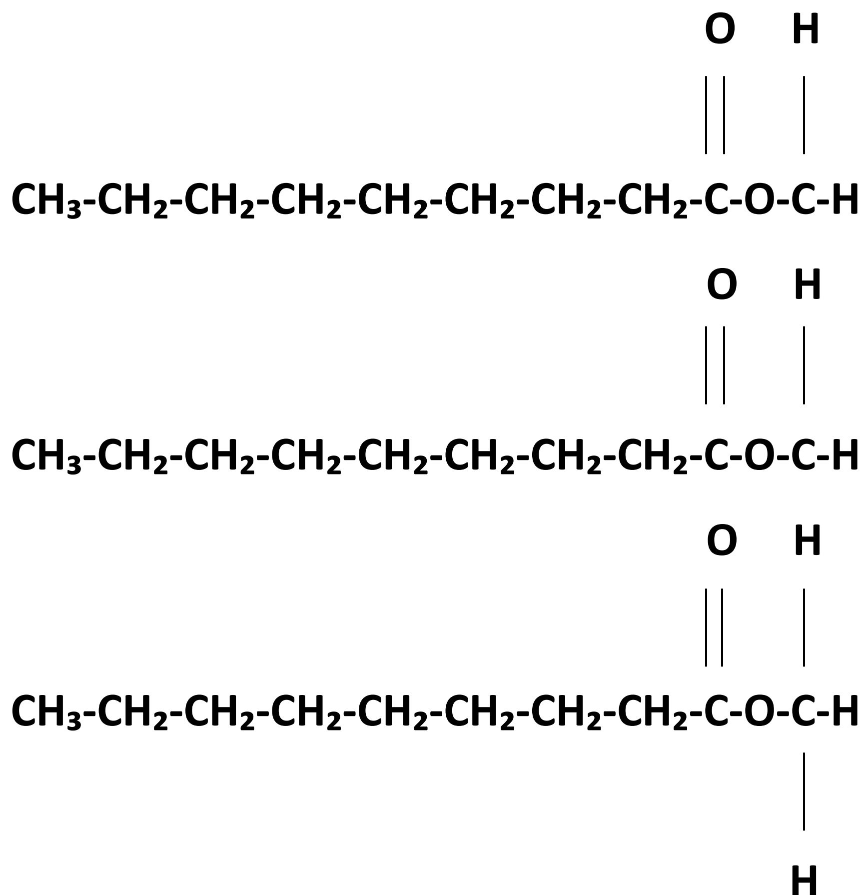

Figure 1-3. Triglyceride structure. Triglycerides are made up of three fatty acids and a glycerol backbone.

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Adipocyte volume changes in response to storage or lipolysis. Adipocytes expand when weight is gained, and shrinks, but do not disappear, when weight is lost. Adipocyte size can be used as a surrogate measure of fatness. Differences in fat cell size also depend on location. In humans, subcutaneous adipocytes are typically larger than visceral adipocytes (Lafontan, 2011). Body mass index (BMI) is an indicator of fatness, but does not measure the percentage of body fat. BMI is calculated in humans by weight divided by height. For humans, a normal BMI is between 18.5 and 24.9, underweight is below 18.5, overweight is between 25 and 29.9, and obese is greater than 30 (Centers for Disease Control and Prevention: Division of Nutrition, 2011).

During energy surplus, such as after a meal, adipocytes participate in energy storage. In response to the postprandial rise in blood glucose levels, the pancreas secretes insulin. Insulin is the primary hormonal mediator of energy storage in adipose tissue. It promotes fat storage by stimulating glucose and fatty acid uptake by the adipocyte, promotes fatty acid and glycerol synthesis, and inhibits lipolysis. Dietary triglycerides are delivered to adipose tissue by chylomicrons, lipoprotein, cholesterol, and triglyceride particles that transport dietary lipids through the circulation. The capillary endothelial cells of the liver and adipose tissue express the protein lipoprotein lipase, which hydrolyzes triglycerides in chylomicrons into fatty acids and glycerol for their uptake into adipocytes. Fatty acids and glycerol re-esterify in the fat cell, forming triglycerides.

During fasting or energy restriction, the pancreas decreases insulin release, and releases glucagon which counters the actions of insulin and promotes fat utilization. The rise in plasma

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glucagon and decrease in insulin activates adipose triglyceride lipase and hormone sensitive lipase (HSL), which promote lipolysis in the adipocytes. Lipolysis is the breakdown of lipids and the hydrolysis of triglycerides into glycerol and free fatty acids. These lipase enzymes hydrolyze fatty acids from triglyceride molecule stores within adipocytes. The fatty acids and the glycerol backbone are released into circulation for metabolism by other tissues or are re-esterified for storage within the adipocytes. Free fatty acids can be taken up by the liver, heart, and skeletal muscle and metabolized for energy.

Plasma levels of non-esterified fatty acids (NEFA) and glycerol levels can be used as indices of lipolysis. NEFA are transported in the blood, attached to serum albumin, to tissues such as the liver, heart and muscle, which oxidize fatty acids for energy through beta-oxidation. Beta-oxidation is the process by which long-chain fatty acids are metabolized to produce acetyl CoA. Acetyl CoA is the entry molecule for the citric acid cycle, where acetyl CoA is oxidized to CO_2 and H_2O in the mitochondria to produce energy in the form of ATP. Sympathetic stimulation of adipose tissue also promotes lipolysis and fatty acid oxidation through actions of the hormone epinephrine. In arctic ground squirrels, serum NEFA concentrations were significantly greater during hibernation ($p < 0.0001$), and least during non-hibernation (Barger et al., 2006). A study in wild black bears found that plasma NEFA increased during hibernation (LeBlanc et al., 2001). Since the bears were not eating during hibernation, the increased NEFA concentrations most likely indicate increased mobilization of lipid reserves from adipose tissue.

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Adipokines

Adipose tissue secretes various protein signals and factors called adipokines (Trayhurn and Wood, 2004). Adipokines are hormones, cytokines or other signaling molecules that affect metabolism, nutrient intake and energy expenditure (Lafontan, 2011). Adipokines vary in function (Trayhurn et al., 2006), and many change in abundance in relation to fatness and adipocyte size. With increasing adipocyte size, there is differential expression of more pro-inflammatory than anti-inflammatory factors (Lafontan, 2011).

Leptin is the canonical adipokine, originally characterized for its role in regulation of food intake and energy expenditure (Maffei et al., 1995). Leptin is primarily secreted by white adipose tissue, but also secreted in smaller amounts by cells in the epithelium of the stomach and in the placenta. Expression of leptin changes in proportion to adipose tissue mass and exerts feedback regulation via the hypothalamus on decreasing food intake, insulin sensitivity and metabolism.

Leptin has been most extensively studied in rodents and humans. The first studies of the effects of leptin were observed in mutant obese mice. These mice had mutations in the ob gene that resulted in a total lack of leptin production, which led to severe obesity. When injections of leptin were given to these mice, their food intake decreased, metabolic rate increased, and they lost weight. Humans with leptin mutations are prone to obesity that can be remedied with leptin treatments (Farooqi et al., 1999), however, the incidences of leptin mutations are infrequent (Friedman, 2011). A study in 64 healthy postmenopausal women

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found that leptin regulated energy balance by inhibiting food intake, and leptin secretion was linked to the quantity and not quality of food intake (Larsson et al., 1998).

Leptin relationships with adiposity have been observed in other species. Plasma leptin measured in spayed female beagles revealed a positive correlation ($r = 0.920$; $p < 0.001$) between plasma leptin levels and body fat (Sagawa et al., 2002). The dogs' feeding and exercise were controlled and there was a range of body weights and body condition scores represented. The results indicated that plasma leptin is a quantitative indicator of adiposity in dogs.

Expression of the leptin receptor (Ob-R) protein in Japanese black bear uteri and ovaries suggest the corpus luteum and endometrium are directly targeted by leptin, and that leptin develops and maintains both the endometrium and ovaries during delayed implantation (Nakamura et al., 2009). The findings also suggest that reproductive organs, such as the uterus and ovary, are directly targeted by leptin during pregnancy in Japanese black bears.

Adiponectin is secreted exclusively from adipose tissue. Adiponectin regulates blood glucose and activates fatty acid oxidation pathways. During hibernation, fatty acids become the main source of energy, therefore adiponectin is of interest because of its role in fat metabolism. Adiponectin secretion has an ultradian rhythm, with decreased concentrations at night and increased concentrations in the early morning. This ultradian rhythm is the same as is seen with cortisol and leptin-binding protein concentration (Gavrila, 2003).

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Adiponectin is negatively correlated with obesity. In obese people and humans with type 2 diabetes, adiponectin levels are decreased (Matsubara et al., 2002). When obese people lose weight, there is a subsequent increase in their adiponectin levels (Yang and Lee, 2001). Women and female rodents have greater concentrations of adiponectin than males (Combs et al., 2004).

Energy utilization and fat mobilization during mammalian hibernation

All hibernating animals experience a metabolic depression and rely on fat stores to support their energy needs during hibernation. Hibernating animals suppress carbohydrate metabolism and increase fatty acid catabolism during hibernation. Bears will enter hibernation in late fall as obese and emerge in the spring as lean, losing 15-20% of their fat mass (Hock, 1960). Only fat mass is lost during hibernation, not lean body mass (Nelson, 1973). Metabolic depression is a critical component of hibernation. Hibernating animals would not be able to survive hibernation if their metabolic rate remained at that of their active period.

Among the principle products of fatty acid catabolism are ketone bodies. Beta-hydroxybutyrate is a major ketone body produced when the body cannot absorb glucose properly (insulin resistance) or experiences prolonged hypoglycemia. Ketone bodies are typically excreted in the urine, but excessive amount of ketones, termed ketosis, leads to metabolic acidosis. However, ketones are better tolerated by hibernating animals because they are used by muscle and brain tissue for fuel during hibernation.

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The primary source of energy for hibernating 13-lined ground squirrels (*Spermophilus tridecemlineatus*) was lipids stored in white adipose tissue. In this species, serum concentrations of glucose and beta-hydroxybutyrate are generally inversely proportional, across a variety of activity states (Andrews et al., 2009). In ground squirrels, both glucose and beta-hydroxybutyrate enter the heart and brain, but there is a preference for beta-hydroxybutyrate in both of these organs during hibernation. In studies on marmots (Tokuyama et al., 1991) and black bears (LeBlanc et al., 2001), beta-hydroxybutyrate increased dramatically during hibernation.

Regulation of food intake in hibernating mammals follows a circannual rhythm, evidenced by consuming large quantities of food prior to hibernation, no food consumption during hibernation, and limited food consumption when emerging from hibernation. Glucose, fatty acids, AMP-activated protein kinase (AMPK), and hormones such as leptin, insulin, and ghrelin are involved in the regulation of food intake by their influence on the action of neurons (Florant and Healy, 2012).

Adipokines in hibernation

Potential hormonal mediators of hibernation physiology and seasonal variation in weight loss include leptin and adiponectin. The hyperphagia period, during which substantial increased food intake occurs with rapid weight gain, requires a temporary suppression of the normal homeostatic mechanisms that balance food intake with lipid storage. In non-

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hibernating animals, an increase in leptin decreases food intake. However, in animals preparing for hibernation and consuming massive quantities of food, circulating leptin concentrations increase as fat mass increases (Rousseau et al., 2003). During hibernation, leptin concentrations decrease dramatically concurrent with the reduction in food intake (Rousseau et al., 2003).

In woodchucks (*Marmota monax*), leptin levels, food intake, and body mass are positively correlated seasonally, with all three peaking in the fall (Concannon et al., 2001). Leptin infusions given post-hibernation to arctic ground squirrels resulted in a reduction in food intake and prevented weight gain, but did not alter energy expenditure (Boyer et al., 1997). Energy expenditure was estimated by measuring resting metabolic rate, body temperature, and locomotor activity.

Serum leptin has been measured in adult female Japanese black bears using a canine-leptin-specific sandwich ELISA validated for Asian black bear leptin (Tsubota et al., 2008). In mated and unmated bears, the serum leptin concentrations were consistently at low levels from May to August, gradually increased from September to October, and then increased dramatically in late November. During the winter, very small amounts of leptin were observed. In hibernating grizzly bears, plasma leptin levels were less during late hibernation than levels in the active summer period and early hibernation, as measured by radioimmunoassay (Gardi et al., 2011). In a study of Japanese black bears, leptin mRNA expression in white adipose tissue was significantly greater in very large adipocytes, as compared to small and medium-sized adipocytes (Nakamura et al., 2008). Their leptin expression and serum leptin concentrations

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increased in late November, while adipocyte size increased in mid-November. The findings suggest that the bears' nutritional condition may be a reflection of their serum leptin concentrations, and that leptin was primarily secreted from white adipose tissue.

When an animal is preparing for hibernation, eating massive amounts of food and becoming fatter, it would be expected for adiponectin levels to be low. In yellow-bellied marmots (*Marmota flaviventris*), adiponectin levels were decreased in late autumn, as the animals entered hibernation, and increased in the summer, as they prepared for hibernation, in both serum concentrations and expression (Florant et al., 2004). There have been no studies of adiponectin in bears.

Molecular regulators for hibernation induced fat loss

In the fasted mammal, conservation of glucose is controlled by the inhibition of the mitochondrial pyruvate dehydrogenase complex (Randle, 1986). The pyruvate dehydrogenase complex transforms pyruvate into acetyl CoA, which is used in the citric acid cycle to release energy in the form of ATP. Pyruvate dehydrogenase kinase isoenzyme 4 (PDK4) is located in the mitochondrial matrix and expression is stimulated by starvation (Wu et al., 2000). PDK4 regulates glucose metabolism by inhibiting the pyruvate dehydrogenase complex by phosphorylating its subunit. This results in a decrease in glucose metabolism.

During hibernation in ground squirrels, PDK4 is upregulated in white adipose tissue, and heart and skeletal muscle (Buck et al., 2002). Ground squirrels active in the fall had significantly

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greater serum insulin levels ($p < 0.05$) and low levels of PDK4 mRNA in white adipose tissue and skeletal muscle. During the winter, the ground squirrels had low levels of serum insulin and significantly greater PDK4 mRNA expression in white adipose tissue and skeletal muscle ($p < 0.05$). There have been no previous studies of PDK4 in bears.

Carbohydrates are the main source of energy during a hibernating animal's active summer and fall months. During this time insulin secretion is increased. Insulin inhibits PDK4 expression, therefore PDK4 is decreased in non-hibernating animals (Figure 1-4). During hibernation, when there is no food intake, there are reduced amounts of circulating insulin, and the animal switches to obtain its energy from free fatty acids. The peroxisome proliferator-activated receptor alpha (PPARα) regulates gene expression in favor of fatty acid beta-oxidation. PPARα is expressed in adipose tissue and activates PDK4 expression during hibernation.

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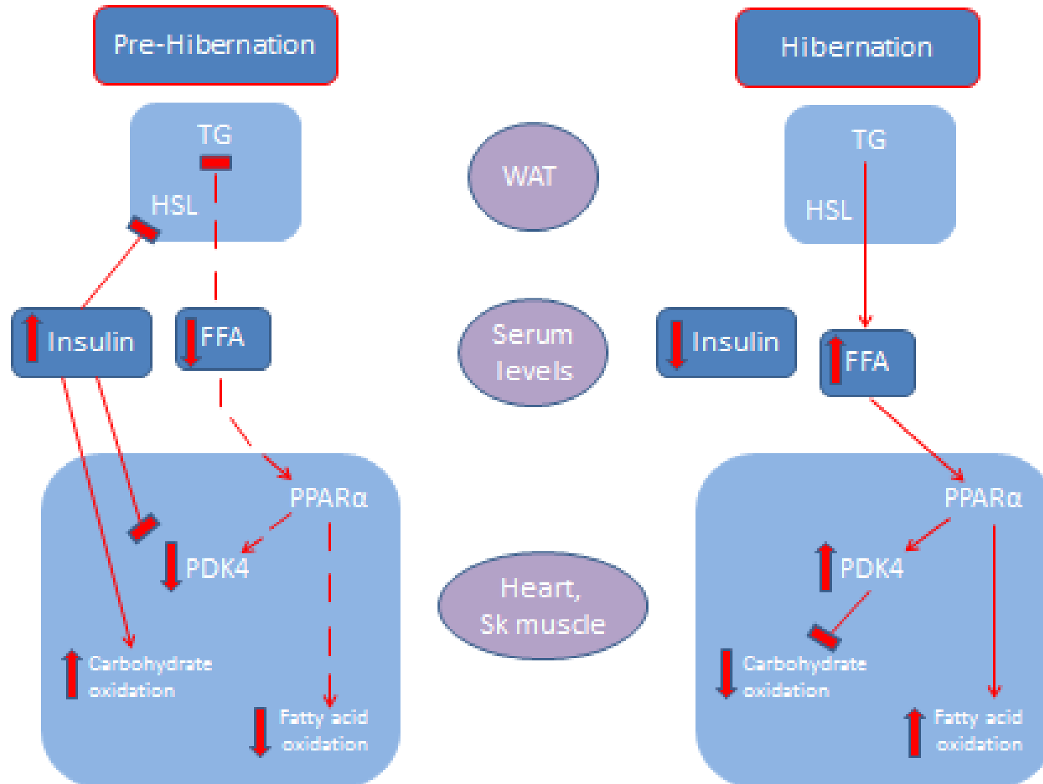

Redrawn based on (Buck et al., 2002)

Figure 1-4. Regulation of PDK4 expression during pre-hibernation and hibernation. This illustrates the switch from carbohydrates to fatty acids as the primary source of fuel during hibernation. Effects of serum levels of insulin and free fatty acids (ffa), secreted from white adipose tissue (WAT), on PDK4 gene expression, and carbohydrate oxidation and fatty acid oxidation in the heart and skeletal muscle in pre-hibernating (September-October) and hibernating (December-January) animals. Solid lines indicate the active mode of regulation and dashed lines indicate secondary regulation. Lines with arrowheads indicate up-regulation/activation and lines with blunt ends indicate down-regulation/inhibition. Abbreviations: HSL, hormone sensitive lipase; PPARα, peroxisome proliferator activated receptor alpha.

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AMP-activated protein kinase (AMPK) is a heterotrimetric enzyme involved in cellular energy homeostasis. AMPK down-regulates ATP-consuming pathways, up-regulates ATP-producing pathways (Carling, 2004), and stimulates glycolysis by increasing glucose uptake in skeletal muscle (Merrill et al., 1997) and the heart (Russell et al., 1999). AMPK is expressed in the liver, brain, and skeletal muscle. AMPK is activated by 5'-AMP and has been recognized as a positive regulator of glycolysis, fatty acid oxidation, and glucose transport in muscle (Lindsley and Rutter, 2004). During hibernation, AMPK may be involved in regulating metabolic energy availability and the switch from utilizing carbohydrates to utilization of free fatty acids (Melvin and Andrews, 2009).

AMPK has three subunits, α, β and γ, and is a functional enzyme highly conserved among animals, from yeast to humans (Kahn et al., 2005). The α subunit is involved in complex formation and has a N-terminal kinase domain and a C-terminal domain (Kahn et al., 2005). The β subunit is able to form complexes with α and γ, and has C-terminal domains (Hudson et al., 2003). The β subunit contains a carbohydrate binding domain, whose function is unknown, but it causes AMPK complexes with glycogen particles (Hudson et al., 2003). The γ subunit has four repeats of a sequence of ~60 residues (Bateman, 1997) and usually forms two tandem domains that bind a molecule of AMP or ATP (Scott et al., 2004). In mouse tissues, quantitative real-time PCR (QRT-PCR) confirmed AMPK α1 is equally dispersed in the liver, kidney, lung, heart and brain, with increased levels in adipose tissue and decreased levels in the spleen and pancreas (Steinberg and Kemp, 2009). AMPK α2 levels are increased in the heart, kidney and

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liver, while small levels are found in the lung, brain and adipose tissue. The pancreas and spleen contain significant AMPK α2 amounts (Steinberg and Kemp, 2009).

In yellow-bellied marmots, an AMPK agonist, 5-aminoimidazole-4-carboxamide 1 B-D-ribofuranoside (AICAR), infused into the 3rd ventricle, induced hibernating marmots to eat during the winter, when they normally do not eat (Florant et al., 2010). The infusion of AICAR-activated AMPK in the arcuate nucleus, caused the marmots to eat during the infusion period and three of the five AICAR-infused marmots had an increase or no change in weight. The four marmots in the control group were given saline infusions and lost weight, consistent with weight loss during hibernation. There have been no studies of AMPK in bears.

Summary

Bears are novel models of energy utilization, specifically fat lay down and fat loss. When hibernating, they are able to decrease their metabolic rate, heart rate and body temperature in order to survive the lack of food availability during the winter. However, they do not have the ability to mimic hibernation responses during the summer. The mechanism of metabolic changes in hibernating bears is currently unknown. The remarkable characteristics of hibernation make the bear a model for understanding food intake and metabolism in humans and other non-hibernating animals.

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CHAPTER II

EXPERIMENTAL DESIGN

Wild and captive American black bears were used for this study. A total of 27 bears were sampled during a period of one year, beginning May 2011 and ending April 2012. None of the bears were sampled repeatedly. Bears were sampled during three seasons, summer, fall and winter, corresponding to the American black bears' three major physiological stages or states (Table 2-1).

Wild bears were provided by the Great Smoky Mountains National Park, Tennessee Wildlife Resource Agency and New Jersey Department of Fish and Wildlife. Wild bears from Tennessee were nuisance bears euthanized for management reasons. Wild bears from New Jersey were immobilized for management reasons, such as tagging and population studies. Captive bears were immobilized for routine clinical examinations. Captive bears all lived in the southern Appalachian region and were provided by Ober-Gatlinburg Municipal Black Bear Habitat (Gatlinburg, TN), Knoxville Zoological Gardens (Knoxville, TN), and Western North Carolina Nature Center (Asheville, NC).

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Table 2-1. The number of wild and captive American black bears evaluated each season.

Season	Wild Bears	Captive Bears
Summer (normal stage) May-August	6	3
Fall (hyperphagic stage) September-November	9	3
Winter (hibernation) December-March	3	3

All bears were immobilized with a combination of ketamine and xylazine (Figure 2-1). Captive bears were typically provided with supplemental oxygen via nasal insufflation. Cervical subcutaneous fat samples were obtained from each bear. A 5 cm x 5 cm area of the dorso-lateral neck was clipped and a sterile surgical preparation performed. The skin was numbed with ~2.0 ml 2% lidocaine administered subcutaneously (s.c.) and a 2.5 cm incision was made through the skin. For most bears, three small pieces of subcutaneous adipose tissue (each approximately 1.3 cm in diameter and 1-5 grams in weight) were obtained. One piece was placed in a 15 ml tube of RNA Later®, one piece was placed in a 15 ml tube of 10% buffered formalin, and one piece was placed in a container of liquid nitrogen or dry ice. RNA Later® is an aqueous tissue storage reagent that stabilizes tissues and protects cellular RNA. We were not able to obtain samples for fixation in formalin and to be frozen from every bear. The skin incision was closed with an absorbable suture and tissue glue. At the end of the procedure, meloxicam, a non-steroidal anti-inflammatory drug (0.1 mg/kg s.c.) and benzathine/procaine penicillin (30,000 IU/kg s.c.) was administered.

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Blood was collected from the femoral or jugular veins and placed into Vacutainer® blood tubes, with and without anticoagulant (heparin). Each captive and live wild bear was measured for body length (snout to tail base, snout to tail tip) and shoulder girth circumference, and body weights were obtained. Captive and live wild bears were then released back into their normal environment following recovery.

Nuisance wild bears were euthanized with an intravenous overdose of a potassium chloride solution or pentobarbitol. Hepatic and abdominal adipose tissue was collected within 20 minutes of death, via an abdominal incision, from all euthanized bears. Tissue samples in RNA Later® were stored at -20 ^{o}C until analyses. Snap frozen tissue samples were stored at -80 ^{o}C until analyses. Tissue samples fixed in formalin were stored in a 4 ^{o}C refrigerator, but the refrigerator was colder than 4°C, and the tissue samples were inadvertently frozen. Heparinized and non-heparinized blood samples were centrifuged, and plasma and serum, respectively, were pipetted into 1.5 ml microcentrifuge tubes, and stored at -80 ^{o}C until analyses.

This study was approved by the University of Tennessee Knoxville Institutional Animal Care and Use Committee (UTK-IACUC) for the use of live vertebrate animals on 7/29/11 (Protocol number 2020-0711). This study was also granted a Scientific Research and Collecting Permit approved by the United States Department of the Interior National Park Service for the Great Smoky Mountains National Park on 8/15/11 (Study number GRSM-01083; Permit number GRSM-2011-SCI-0078).

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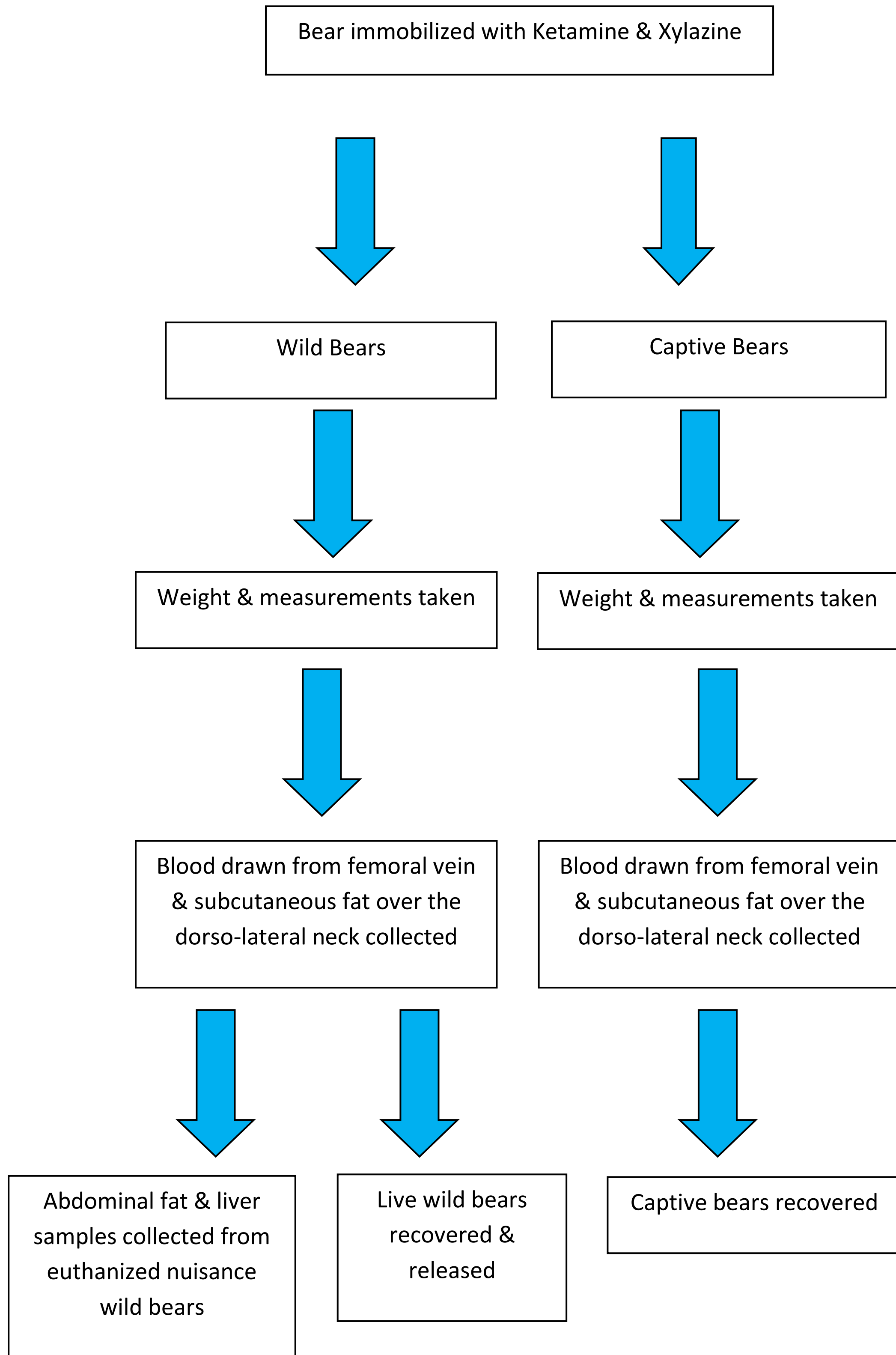

Figure 2-1. Research flow chart for wild and captive bears.

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CHAPTER III

METHODS

Plasma and serum were frozen within four hours of collection and stored at -80 ^{o}C until analyses. Immediately prior to analyses, plasma and serum samples were thawed on ice.

Triglyceride assay

Bear triglycerides were measured using the L-Type Triglyceride M assay kit by Wako Diagnostics (www.wakodiagnostics.com). Briefly, 4 µl of bear plasma, and calibrator, control, and/or deionized (DI) water (for reagent blank) were pipetted into a 96 well microplate. First, 90 µl of Reagent 1 was added to each well, mixed by gentle rotation, and the microplate was covered and incubated at 37 ^{o}C for 5 minutes. The absorbance of each well was measured at 600 nm and this measurement served as the 'sample blank'. Afterward, 30 µl of Reagent 2 was added to each well, mixed by gentle rotation, and the microplate covered and incubated at 37 ^{o}C for 5 minutes. The absorbance of each well was measured at 600 nm and served as the sample absorbance. The final absorbance was calculated by subtracting the sample absorbance from the 'sample blank'. The absorbance of the calibrators vs. concentration was plotted to create a standard curve.

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Non-esterified fatty acid assay

Bear non-esterified fatty acids (NEFA) were measured using the HR Series NEFA-HR (2) assay kit by Wako Diagnostics (www.wakodiagnostics.com). Briefly, reagents were prepared according to package instructions. In separate wells, 3 µl of bear serum, standards and/or DI water were pipetted into a 96 well microplate, and 130 µl of color Reagent A solution was added to each well. The microplate was mixed and incubated at 37 ^{0}C for 5 minutes. The absorbance of each well at 550 nm was measured and served as the 'sample blank.' Afterward, 65 µl of color Reagent B solution was added to each well, the microplate was mixed, and incubated at 37 ^{0}C for 5 minutes. The absorbance of each well at 550 nm was measured and served as the standard absorbance. The final absorbance was obtained by subtracting the 'sample blank' absorbance from the standard absorbance. The final absorbance vs. concentration was plotted to obtain a calibration curve.

Beta-hydroxybutyrate assay

Bear beta-hydroxybutyrate concentrations were measured using the BioVision beta-hydroxybutyrate assay (β-HB) kit (www.biovision.com). Briefly, reagents were prepared according to package instructions. A standard curve was prepared by diluting the β-HB standard to 1.0 mM and adding 10 µl of the standard to 90 µl of distilled water and mixing. Various volumes consisting of 0, 4, 8, 12, 16 and 20 µl were then added to a series of wells. The volume was adjusted to 50 µl/well with assay buffer to generate 0, 4, 8, 12, 16 and 20 nmol per

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well of β-HB standard. Samples were prepared by adding 5 µl of bear serum to 50 µl of reaction mix in a 96 well microplate. The microplate was incubated at room temperature for 30 minutes and protected from light. Samples were measured at 450 nm with Gen5 Data Analysis Software. The background was corrected by subtracting the 0 β-HB control from the standard and control readings. A standard curve nmol/well vs. standard readings was plotted.

Leptin RIA

Bear leptin was measured using a multi-species leptin RIA kit by Millipore (www.millipore.com). The assay utilized ^{125}I-labeled human leptin and guinea pig anti-multi-species leptin antibody to determine the level of leptin in bear serum by the double antibody/polyethyleneglycol (PEG) precipitation technique. On day one, 300 µl of assay buffer was pipetted to the non-specific binding tubes 3-4, 200 µl to the reference (B_0) tubes 5-6, and 100 µl to tubes 7 through end of assay. Then 100 µl of standards, quality controls, and bear serum were added. All standards, controls and sera were run in duplicate. Afterward, 100 µl of multi-species leptin antibody was pipetted to all tubes except total count tubes 1-2 and non-specific binding tubes 3-4. All tubes were vortexed, covered, and incubated overnight at 4 ^{o}C. On day two, 100 µl of ^{125}I-human leptin was pipetted into each tube, and the tubes vortexed, covered, and incubated overnight at 4 ^{o}C. On day three, 1.0 ml of cold (4 ^{o}C) precipitating reagent was added to all tubes except total count tubes 1-2. The tubes were vortexed, and incubated 20 minutes at 4 ^{o}C. All tubes except total count tubes 1-2 were centrifuged at 4 ^{o}C

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for 20 minutes at 2,000-3,000 xg. The supernatant was decanted immediately from all tubes except total count tubes 1-2, the tubes were drained for 15-60 seconds, and excess liquid was blotted from the lip of the tubes. All tubes were counted in a gamma counter for 1 minute. The ng/ml human equivalent of leptin in unknown samples was calculated using automated data reduction procedures.

Adiponectin ELISA

Bear adiponectin was measured using a mouse/rat adiponectin ELISA kit by B-Bridge International, Inc. (www.b-bridge.com). Briefly, reagents were prepared according to package instructions. A 96 well plate was filled with wash solution (~350 µl/well), the plate was aspirated, and inverted to remove the liquid, and 100 µl of adiponectin standard was added to each antibody-coated well. The plate was covered with a plate sealer and incubated at 22-28 °C for 1 hour. The plate was washed by aspirating the liquid, filling each well with wash solution (~350 µl/well), and aspirating the liquid again. Washing was repeated two more times. First, 100 µl of Biotinylated Secondary Antibody Solution was added to each well, the plate was covered, and incubated at 22-28 °C for 1 hour, and the wash procedure was repeated. Second, 100 µl of HRP-Conjugated Streptavidin Solution was added to each well, the plate was covered, and incubated at 22-28 °C for 1 hour, and the wash procedure was repeated. Third, 100 µl of Substrate Solution was added to each well, the plate was covered, and incubated at 22-28 °C

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for 15 minutes. Afterward, 100 µl of Stop Solution was added to each well and the plate was read immediately at 450 nm using a plate reader.

Adipocyte size measurement

Abdominal and subcutaneous adipose tissues were immediately fixed in formalin after collection. The samples were embedded in paraffin, cut into 5 µM sections, and stained with hematoxylin and eosin. Tissue sections were viewed at 10X magnification and images were obtained. The illumination of the images were adjusted using a Nikon® Eclipse E800 microscope and NIS-Elements imaging software program. Adipocytes were measured for each image. Approximately 200 adipocytes per tissue were measured.

RNA isolation of bear adipose tissue

RNA isolation was performed on bear subcutaneous and abdominal white adipose tissue using the RNeasy Lipid Tissue Mini Kit™ by Qiagen (www.qiagen.com). Adipose tissue was stored in RNA Later® solution and frozen within 4 hours of harvesting at -20 °C. At the time of RNA isolation, the sample adipose tissue was thawed on ice, weighed to about 30 mg, and was placed in a 2.0 ml microcentrifuge tube with 1 ml of QIAzol Lysis Reagent. The tissue was completely disrupted using a homogenizer probe for 30-60 seconds and was left at room temperature for 5 minutes. Then 200 µl of chloroform was added and the mixture was shaken

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vigorously for 15 seconds. After 2-3 minutes at room temperature, samples were centrifuged (12,000 x g for 15 minutes at 4 °C) and the upper, aqueous phase was transferred into a new tube, combined with 1 volume (approximately 600 µl) of 70% ethanol and mixed by vortexing. Up to 700 µl of the sampled was loaded onto an RNeasy Mini spin column and placed in a 2 ml collection tube and centrifuged for 15 seconds at 10,000 rpm. After the flow-through was discarded, the sample was washed with 700 µl of Buffer RW1 and 500 µl of Buffer RPE, and then centrifuged for 15 seconds after each was added and the flow-through discarded. The last wash consisted of adding 500 µl of Buffer RPE, centrifuged for 2 minutes, and the flow-through discarded. The longer centrifugation dried the spin column membrane, so that there was no ethanol carried over during the RNA elution. The RNeasy spin column was placed in a new 2 ml collection tube and centrifuged for 1 minute to eliminate any carryover of Buffer RPE. The RNeasy spin column was placed in a new 1.5 ml collection tube and 50 µl of RNase-free water was added to the spin column membrane. The sample was centrifuged for 1 minute to elute the RNA. The eluate was then placed directly in the RNeasy spin column and the sample was centrifuged for 1 minute. The RNA samples were frozen at -80 °C.

RNA isolation of bear liver

RNA isolation was performed on bear liver using the RNeasy Mini Kit™ (Qiagen). Liver samples were stored in RNA Later® solution and frozen within 4 hours of harvesting at -20 °C. At the time of RNA isolation, the liver samples were thawed on ice and weighed to about 30

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mg. Before the procedure began, 10 µl of Beta-Mercaptoethanol (β-ME) was added per 1 ml of Buffer RLT and vortexed. The liver samples were added to 600 µl of β-ME/Buffer RLT solution in a 1.5 ml microcentrifuge tube. The samples were disrupted using a homogenizer probe for approximately 20-40 seconds and then the lysate was centrifuged for 3 minutes at 10,000 rpm. The supernatant was removed and transferred to a new 1.5 ml microcentrifuge tube. Approximately 1 volume (600 µl) of 50% ethanol was added to the cleared lysate and mixed by pipetting. Up to 700 µl of the sample was transferred to an RNeasy spin column and placed in a 2 ml collection tube. The sample was centrifuged for 15 seconds and the flow-through was discarded. Then 350 µl of Buffer RW1 was added to the RNeasy spin column, centrifuged for 15 seconds and the flow-through discarded. Approximately 10 µl of DNase I stock solution was added to 70 µl of Buffer RDD, mixed gently by inverting the tube and centrifuged. The 80 µl of incubation mix was added to the RNeasy spin column membrane and sat at room temperature for 15 minutes. Then 350 µl of Buffer RW1 was added to the RNeasy spin column, centrifuged for 15 seconds and the flow-through was discarded. Next, 500 µl of Buffer RPE was added to the RNeasy spin column, centrifuged for 15 seconds to wash the spin column membrane and the flow-through discarded. Another 500 µl of Buffer RPE was added to the RNeasy spin column, centrifuged for 2 minutes and the flow-through discarded. The longer centrifugation dried the spin column membrane so that no ethanol was carried over during RNA elution. The RNeasy spin column was placed in a new 2 ml collection tube and the RNeasy spin column was centrifuged for 1 minute. The RNeasy spin column was placed in a new 1.5 ml collection tube and 50 µl of RNase-free water was added to the spin column membrane. The RNeasy spin

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column was centrifuged for 1 minute to elute the RNA. The eluate was then pipetted into the spin column membrane and centrifuged for 1 minute. The RNA samples were frozen at -80 °C.

Spectrophotometer

The concentration of bear RNA from adipose tissue and liver was determined using a UV/Visible Spectrophotometer (Ultrospec 3100 Pro; Amersham Biosciences). Approximately 96 µl of RNase-free water and 4 µl of RNA was placed in a 1.5 ml microcentrifuge tube and mixed by vortexing. The sample was then placed into the cuvet of the Spectrophotometer with a dilution factor of 25. A water reference sample was first evaluated, then ratio, concentration, 260 nm and 280 nm from RNA were measured in µg/ml. After blanking on water, absorbance was read at 260 nm and 280 nm. Ratio of 260:280 was used to assess quality concentration (ug/ml) and calculated based on OD_{260}. The criteria for "good quality" RNA was a 260:280 ratio of at least 1.60.

Reverse transcription

Reverse transcription (RT) was performed on 100 ng of bear adipose tissue and liver RNA using the iScript cDNA Synthesis Kit™ (Bio-Rad Laboratories, Inc.; www.bio-rad.com). The components of each RT reaction included 4 µl of 5x iScript reaction mix, 1 µl of iScript reverse transcriptase, and nuclease-free water and RNA template, which were determined upon each

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RNA concentration sample. The total volume of the reaction was 20 µl. The samples were mixed by vortexing and then placed in a RT-PCR machine (Eppendorf Mastercycler). The reaction protocol was 5 minutes at 25 °C, 30 minutes at 42 °C, 5 minutes at 85 °C, and then held at 4 °C.

Gel electrophoresis

Gel electrophoresis was performed to verify that there was not any RNA degradation of the bear adipose tissue and liver samples at 28S and 18S bands (Figure 3-1A), and to verify that RT was successful for the bear adipose tissue and liver samples (Figure 3-1B). To make a 1% agarose gel, 1.0 grams of agarose was added to a mixture of 98 ml of dH_2O and 2 ml of 50 xTAE. The mixture was heated in a microwave for approximately 1-2 minutes, 4 µl of Ethidium bromide was added and the mixture was cooled by sitting out on laboratory bench top. The mixture was poured into a gel electrophoresis apparatus, a comb was added, and the mixture sat out for approximately 30 minutes to form an agarose gel. The agarose gel was put into a buffer-filled box supplied with electrical current. A mixture of cDNA from PCR was added to about 10 ml of 6x loading dye, mixed by vortexing, and added to the appropriate wells in the agarose gel. The agarose gel was run for approximately 45 minutes at 100 volts. A picture of the gel was taken with FOTO/Analyst® PC Image software.

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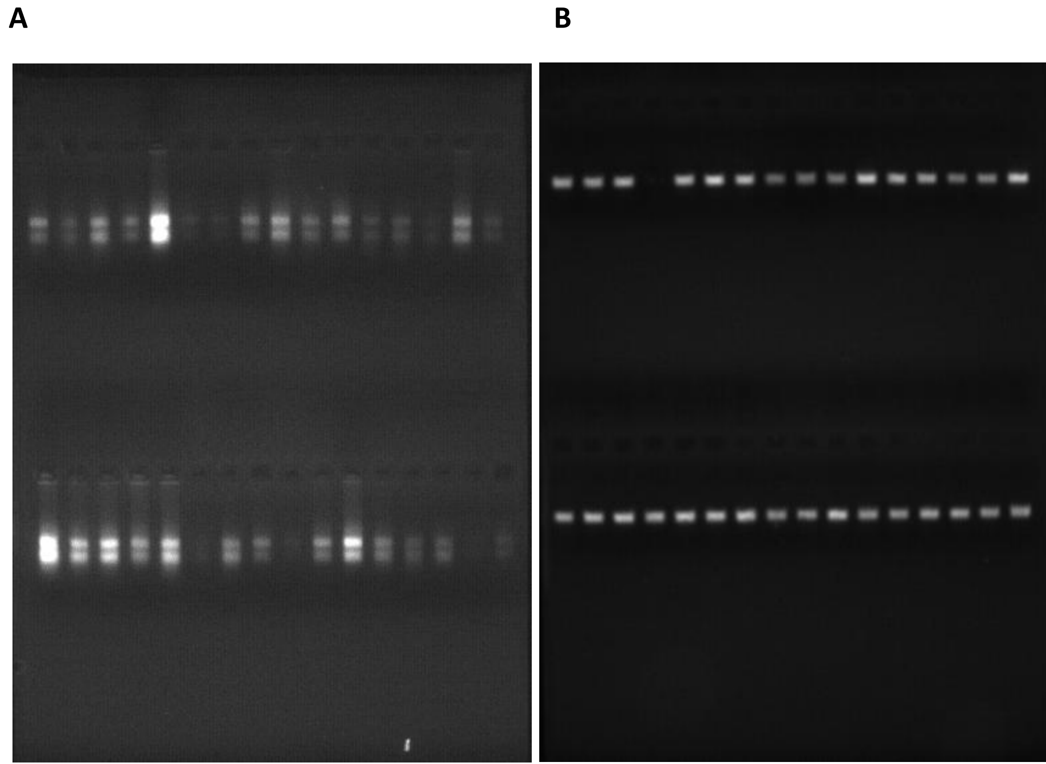

Figure 3-1. Gel electrophoresis. (A) RNA quality check for intact 18S and 28S bands and (B) Confirming integrity of reverse transcription for QPCR. Reverse transcription was followed by PCR for giant panda primer ribosomal protein S14 (RPS14). Bands seen on the gel represent that RNA was isolated (A) and that reverse transcription was successful (B).

Primer design and DNA sequencing

Primers for leptin, adiponectin and PDK4 for QPCR were designed against the giant panda (*Ailuropoda melanoleuca*) genome because the black bear genome is only a draft sequence, and there is little information on the Ursidae bear family genome. The giant panda genome is the closest related genome to the black bear in the UCSC Genome Bioinformatics browser (www.genome.ucsc.edu). The giant panda species had split off from the Ursus bear genus around 12 million years ago (Yu et al., 2007). Primers for protein kinase, AMP-activated,

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alpha 1 catalytic subunit (PRKAA1) for QPCR were designed against the human (*Homo sapiens*) genome. An isomer of AMPK, PRKAA1, primers was designed for this study. Genes of interest were searched using Ensembl (www.ensembl.org), which provided the cDNA sequence for the gene of interest. The cDNA sequence was then used as a template for primer design using Primer3 (www.frodo.wi.mit.edu/Primer3).

Several criteria had to be met for designing primers (Table 3-1). First, the product size had to be less than 250 base pairs (bp). Second, the forward and reverse sequences had to have about the same melting temperature. Third, the GC% content (the number of guanine (G) and cytosine (C) in the primer as a percentage of total bases) for the forward and reverse sequences had to both be around 50 to be efficient primers. Lastly, the forward and reverse sequences needed to be on separate exons. This was done so that there was no accidental amplification of genomic DNA due to the limitations of product size.

After primers were designed and PCR was performed, the band containing bear cDNA with the primer of interest was detected on an agarose gel. The band was removed from the gel and a gel extraction procedure was performed. The gel bands were then submitted to the DNA Sequencing Lab at The University of Tennessee for identification of the DNA sequence of the gene. Once the DNA sequence was identified, the forward and reverse sequences were entered into UCSC Genome Bioinformatics (www.genome.ucsc.edu) for verification.

PCR conditions were optimized for each primer by adjusting cycling conditions and running a temperature gradient to find the optimal C_t value (Table 3-2). Temperature was

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adjusted in the denaturation, annealing and extension steps from 54 °C to 64 °C. Optimal temperature for PCR was obtained by locating the highest C_t value at the highest temperature in the PCR output, and re-running with that temperature. This also had to result in a single melt peak, to confirm that there was only one PCR product.

After testing multiple housekeeping primers with bear cDNA, mouse primer 18S ribosomal RNA (designed by QuantiTect) was used as the housekeeping gene because it exhibited detectable and stable expression across all samples and because it had a standard curve with good efficiency. Beta-tubulin and histidine triad nucleotide binding protein 1 (HINT1) were designed against the giant panda genome as possible housekeeping genes, but neither were detectable on an agarose gel when run on PCR with bear cDNA. Ribosomal protein S14 (RPS14) was designed against the giant panda genome as a possible housekeeping gene and was detectable on an agarose gel with bear cDNA, but the C_t values were too low on QPCR. Mouse primer ribosomal protein L13 (RPL13) (designed by QuantiTect) was detectable on an agarose gel with bear cDNA, but several bears had very low C_t values that were not detectable.

Table 3-1. Design of QPCR primers.

Primer	Forward Sequence	Reverse Sequence	Product Size
Panda Leptin	GCTTTGGCCCTATCTGTCCT	TGCAGACTGGTGAGGATCTG	237 bp
Panda Adiponectin	CCTGGTGAAAAGGGTGAGAA	CAATCCCACACTGAATGCTG	174 bp
Panda PDK4	GAGACGAGAAATTGGCAAGC	GAGATCATTGAGCGCCTCTT	117 bp
Human PRKAA1	GGAACCCTTCCATTTGATGA	TATGATGGATCCTCGGGAAA	221 bp

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Table 3-2. Cycling conditions of QPCR primers.

Primer	Cycling Conditions
Panda Leptin	1. 95.0 °C for 2 min. 2. 95.0 °C for 45 sec. 3. 58.0 °C for 45 sec. 4. 72.0 °C for 45 sec. 5. Go to #2, 39 more times 6. Melt curve 55.0 °C to 95.0 °C, increment 0.5 °C for 5 sec.
Panda Adiponectin	1. 95.0 °C for 15 min. 2. 57.9 °C for 1 min. 3. 95.0 °C for 15 sec. 4. 57.9 °C for 30 sec. 5. 72.0 °C for 30 sec. 6. Go to #3, 39 more times 7. Melt curve 55.0 °C to 95.0 °C, increment 0.5 °C for 5 sec.
Panda PDK4	1. 95.0 °C for 15 min. 2. 57.9 °C for 1 min. 3. 95.0 °C for 15 sec. 4. 57.9 °C for 30 sec. 5. 72.0 °C for 30 sec. 6. Go to #3, 39 more times 7. Melt curve 55.0 °C to 95.0 °C, increment 0.5 °C for 5 sec.
Human PRKAA1	1. 95.0 °C for 15 min. 2. 57.9 °C for 1 min. 3. 95.0 °C for 15 sec. 4. 57.9 °C for 30 sec. 5. 72.0 °C for 30 sec. 6. Go to #3, 39 more times 7. Melt curve 55.0 °C to 95.0 °C, increment 0.5 °C for 5 sec.

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Polymerase chain reaction

Polymerase chain reaction (PCR) was performed on bear adipose tissue and liver RNA using the RT-PCR machine (Eppendorf Mastercycler) after RT was done to amplify the cDNA gene fragments for sequencing. Table 3-3 lists the materials used for PCR.

The samples were mixed by vortexing and placed in a RT-PCR machine (Eppendorf Mastercycler). The reaction protocol was 2 minutes at 94 °C, 36 cycles of 45 seconds at 94 °C, 45 seconds at 58 °C, and 45 seconds at 72 °C, and 5 minutes at 72 °C.

Table 3-3. PCR materials.

10x PCR Buffer (-$MgCl_2$)	5.0 µl
$MgCl_2$	1.5 µl
dNTP	1.0 µl
pTAQ	0.2 µl
DNase-free water	38.3 µl
Forward Primer	0.5 µl
Reverse Primer	0.5 µl
cDNA	5.0 µl

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Quantitative polymerase chain reaction

Bear cDNA was run on a Quantitative Polymerase Chain Reaction (QPCR) machine (CFX96™ Real-Time System; C1000™ Thermal Cycler; Bio-Rad) to observe the changes in mRNA expression in the adipokines of interest. Expression was quantified using QuantiTect SYBR Green PCR kit by Qiagen. QPCR was performed on a 96 well microplate, using 20 µl per well. Each well contained 10 µl of Master Mix 2x SYBR Green, 7 µl of nuclease-free water, 1 µl of the forward primer, 1 µl of the reverse primer, and 1 µl of 1:10 dilutions of cDNA.

Statistical analyses

A completely randomized design (CRD) was performed on the data using ANOVA to statistically test for main effects of season and of status (wild or captive) or adipose depot (subcutaneous or abdominal, relevant for the adipocyte size data) on each of the measured traits. An interaction term (season X status) was used to test for effects of season that varied according to status. Differences in leptin, adiponectin, PDK4, PRKAA1, glucose, triglycerides, non-esterified fatty acids, beta-hydroxybutyrate, adipocyte size and BMI were measured. A p-value of <0.05 was considered significant. Results are shown as means $\pm$ standard error of the mean. Fisher's protected least significant difference (LSD) was used when ANOVA resulted in a p-value of <0.05, and was used to compared statistical differences within season between wild and captive bears.

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CHAPTER IV

RESULTS & DISCUSSION

Results

Variation in body mass

A modified body mass index (BMI) score was calculated by dividing the bears' body weight by its length (snout to tail base) and was used as an index of adiposity.

Captive bears' mean BMI was significantly greater than that of wild bears' in each of the three sampling seasons ($p < 0.0001$; Figure 4-1). These differences are not surprising due to differences in food availability and opportunity for exercise. All captive bears were fed a diet of commercial dry dog food (kibble), and small amounts of assorted fruits and vegetables. Food was provided daily, year round, to the Knoxville Zoo bears (all fall captive bears) and daily during the summer and fall to the Ober-Gatlinburg Municipal Black Bear Habitat and Western North Carolina Nature Center bears (summer and winter captive bears). In contrast, nearly all of the wild bears' primary mid to late summer forages (e.g. blueberries, huckleberries and cherries) and fall forages (e.g. acorns) were scarce during 2011 in the Smoky Mountains (Bill Stiver, Great Smoky Mountains National Park (GSMNP), personal communication). The wild bears continue to lose weight in the early summer until the huckleberries and blueberries are available (Bill Stiver, personal communication). As a result, the wild GSMNP bears were much leaner than the other bears sampled (Figure 4-2). Additionally, the older and larger bears in the GSMNP were forcing the younger and smaller bears out of the food areas and into the campsite

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areas (Bill Stiver, personal communication). Summer and fall 2011 proved to be a hard time for the GSMNP, with over a dozen nuisance bears euthanized. During a more typical summer and fall, only one or two nuisance bears are euthanized (Bill Stiver, personal communication). Ten of the wild bears (6 summer and 4 fall wild bears) studied were GSMNP nuisance bears euthanized by the National Park Service for repeatedly damaging campsites and automobiles in search of food.

Captive bears evaluated during the fall had slightly, but not significantly, greater BMI values than captive bears during the summer or winter seasons, as would be expected (Figure 4-3). All captive fall bears were from the Knoxville Zoo, and keepers reported the bears do act hungrier in the fall. Typically, keepers will give these bears some extra pieces of fruit or vegetable in the fall, to keep the bear from exhibiting unwanted behaviors, such as pacing. These captive fall bears, however, eat less food than a wild bear during this time. Knoxville Zoo bears are allowed to stay awake year round (they do not hibernate). During the winter, their keepers report that the bears eat less food than they do in the summer or fall, although they are offered the same diet year round.

The wild winter bears had a greater, but not significantly greater, BMI than wild bears sampled in summer and fall. This is not consistent with the expected weight loss during hibernation. However, all of the wild winter bears were lactating females from New Jersey and not nuisance GSMNP bears (Figure 4-4). This confounds evaluating the effect of season on body mass. There were also no significant differences across seasons for wild or captive bears.

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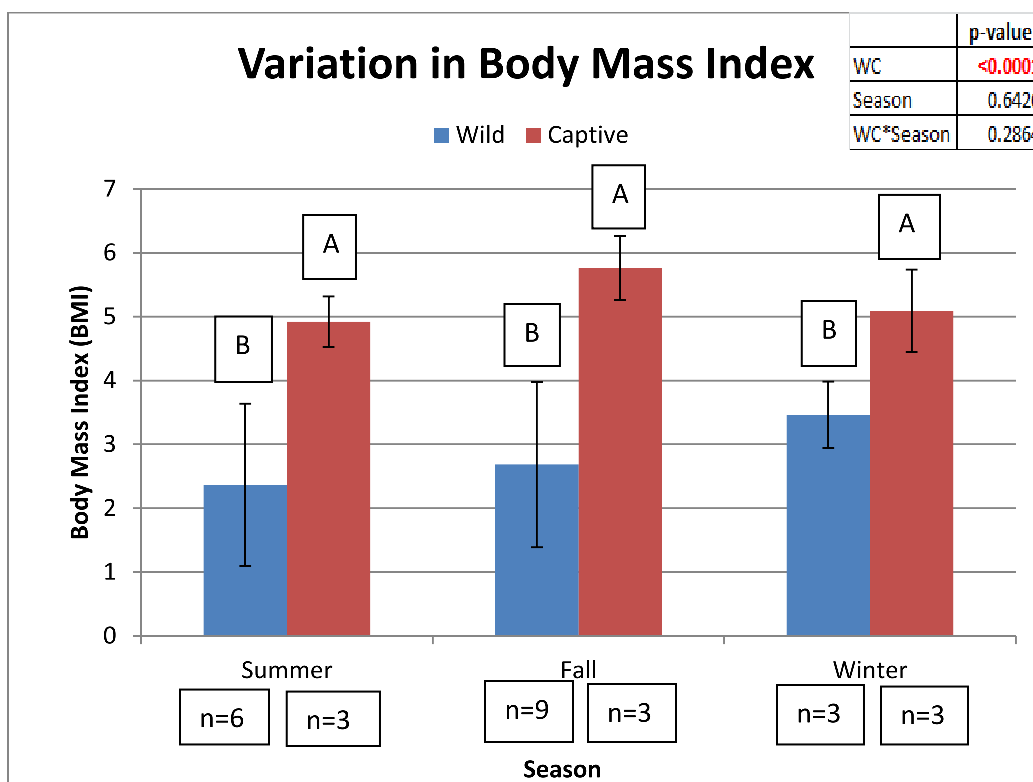

Figure 4-1. Variation in body mass index (BMI) of wild and captive bears captured during the summer, fall and winter seasons. BMI was calculated by body weight/total length. Statistical comparison was for captive vs. wild bears within each season. Groups with the same superscripts are not significantly different from each other. Abbreviation: WC, wild vs. captive.

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Figure 4-2. Euthanized nuisance American black bear from the Great Smoky Mountains National Park, Gatlinburg, TN, being weighed during the summer. This bear weighed 134 pounds and had a body mass index of 2.39.

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Figure 4-3. Immobilized captive American black bear being weighed during the summer. This bear weighed 384 pounds and had a body mass index of 5.65.

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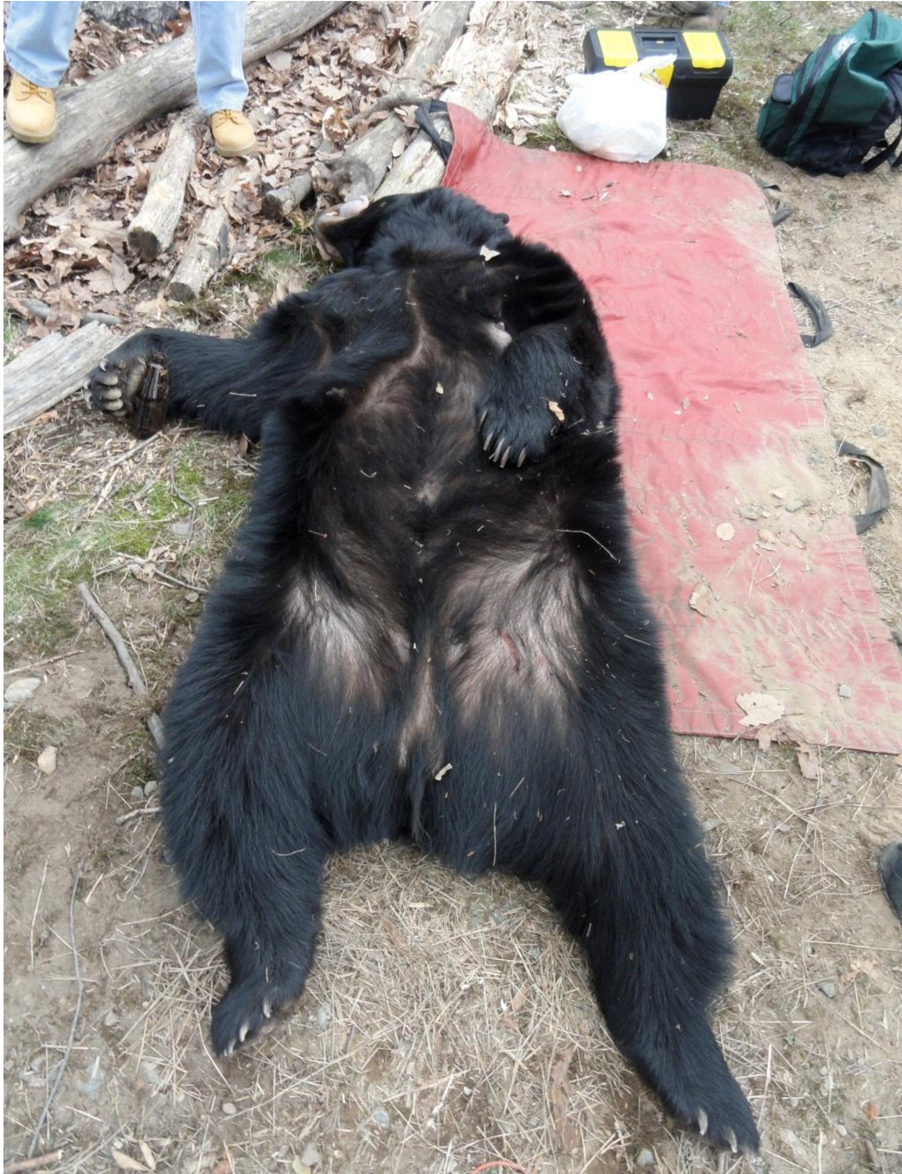

Figure 4-4. Immobilized lactating female wild American black bear in New Jersey being weighed during the winter. This bear weighed 268 pounds and had a body mass index of 4.39.

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Serum and plasma assays

Plasma triglycerides

Plasma triglyceride concentrations varied considerably within the wild bear group (Figure 4-5). There were no statistically significant differences between wild vs. captive bears or across seasons. There was also no significant interaction effect of status (wild vs. captive) and season for plasma triglycerides.

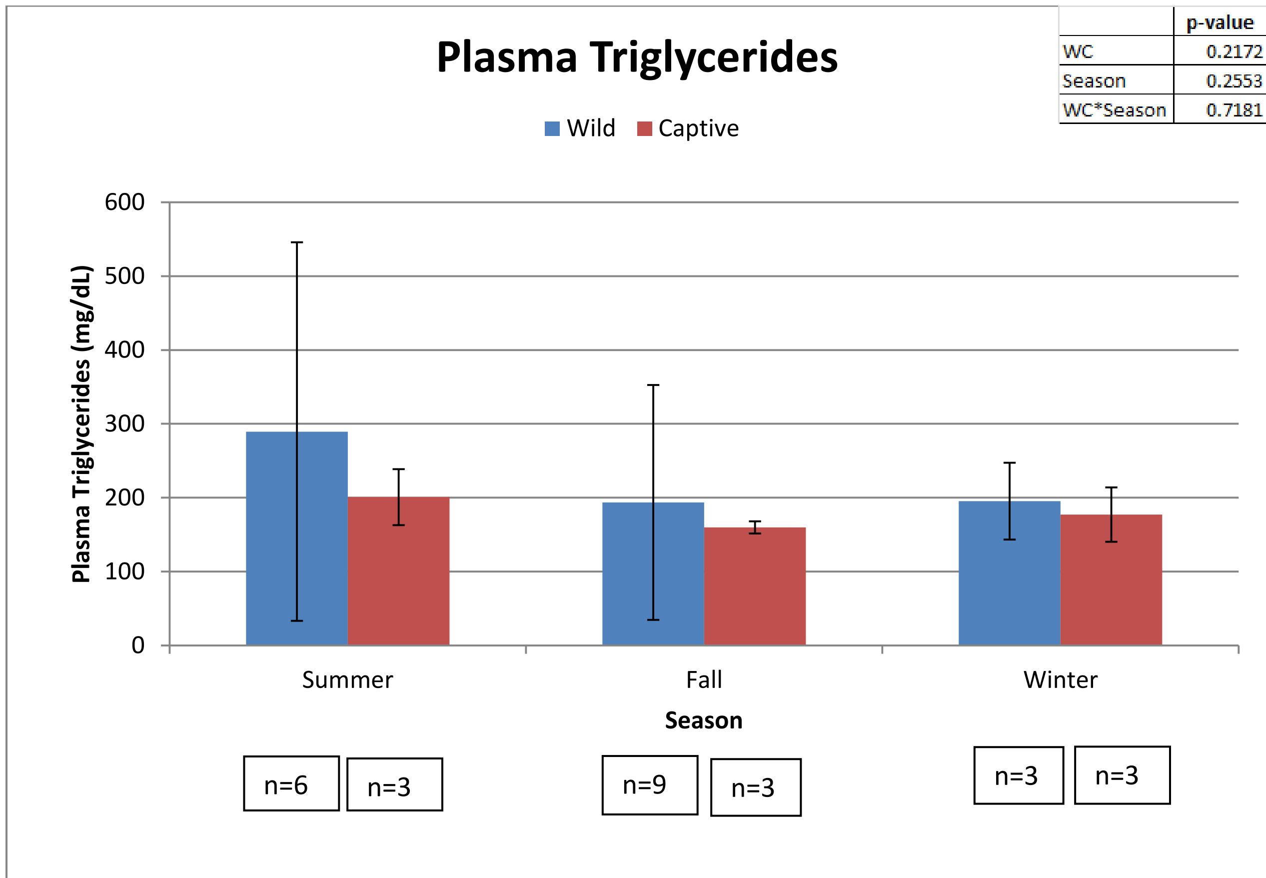

Figure 4-5. Mean (± SEM) American black bear plasma triglycerides measured by assay. Abbreviation: WC, wild vs. captive.

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Non-esterified fatty acids

Plasma NEFA levels, used as an index of lipolysis, were not statistically different between wild vs. captive bears or across seasons (Figure 4-6). There was also no significant interaction effect of status and season for plasma NEFA.

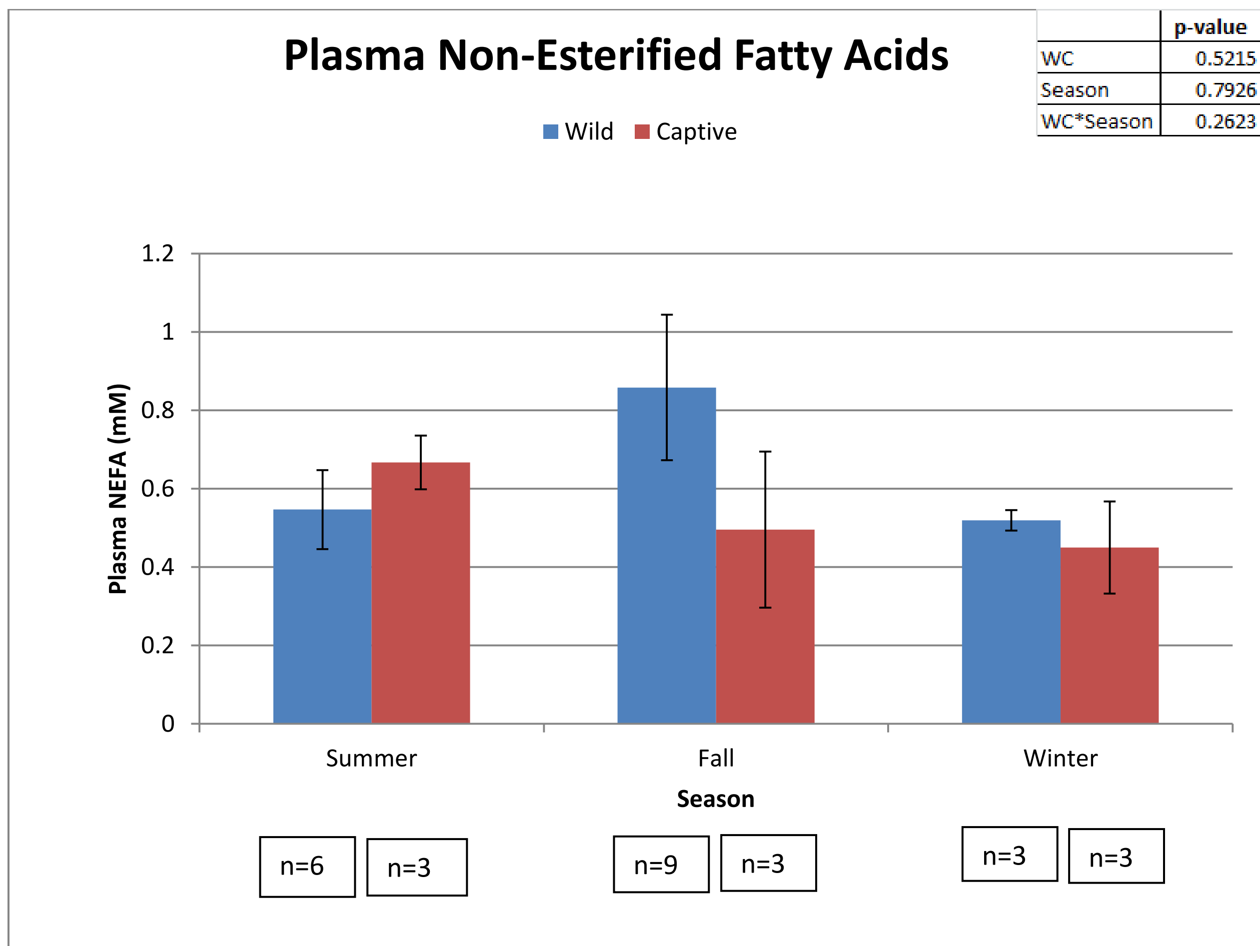

Figure 4-6. Mean (± SEM) American black bear plasma non-esterified fatty acids measured by assay. Abbreviation: WC, wild vs. captive.

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Beta-hydroxybutyrate

Serum beta-hydroxybutyrate concentrations were significantly greater in wild vs. captive bears in each season (p = 0.0099; Figure 4-7), suggesting higher rates of fatty acid oxidation in wild bears. There was no statistically significant effect of season. There was also no significant interaction effect of status and season in captive bears. Wild winter bears showed greater concentrations than wild fall bears, based on Fisher's protected LSD.

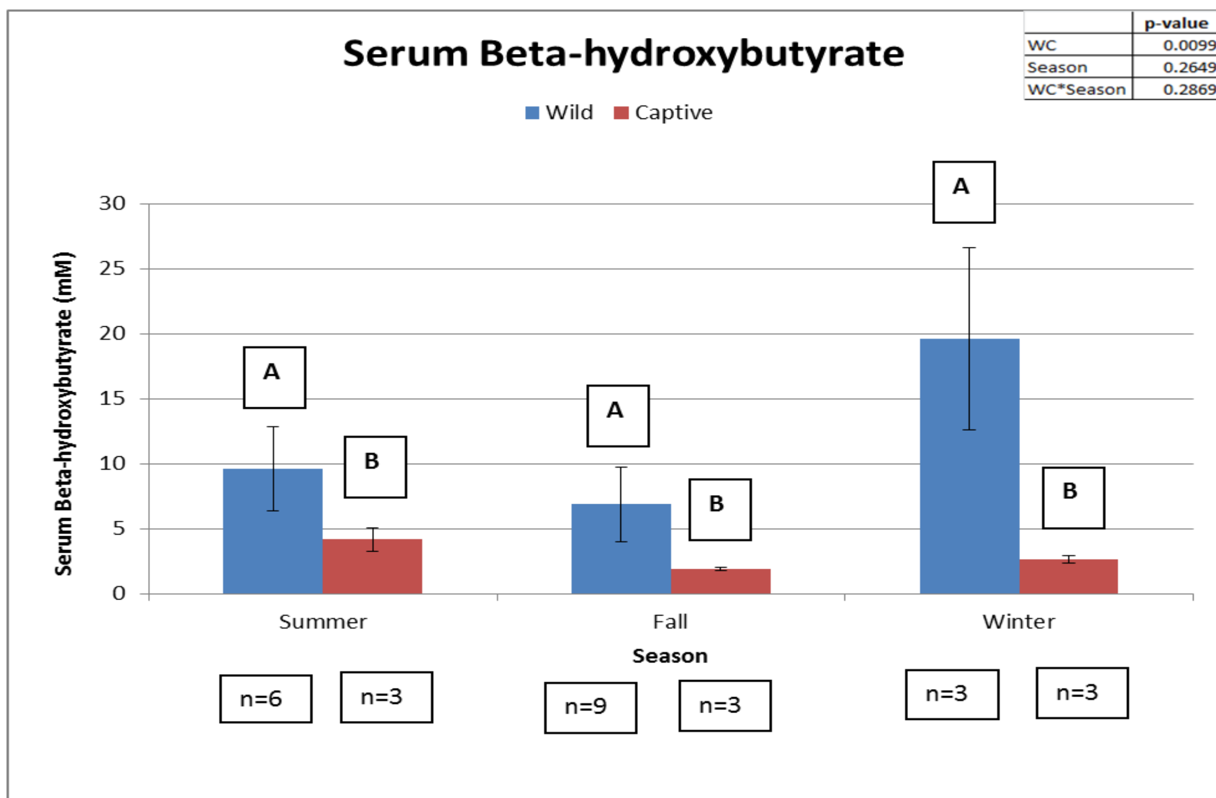

Figure 4-7. Mean (± SEM) American black bear serum beta-hydroxybutyrate measured by assay. Groups with the same superscripts are not significantly different from each other. Abbreviation: WC, wild vs. captive.

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Leptin

Plasma leptin concentrations did not differ significantly between wild vs. captive bears or across seasons (Figure 4-8). There was also no significant interaction effect of status (wild vs. captive and season) for serum leptin levels.

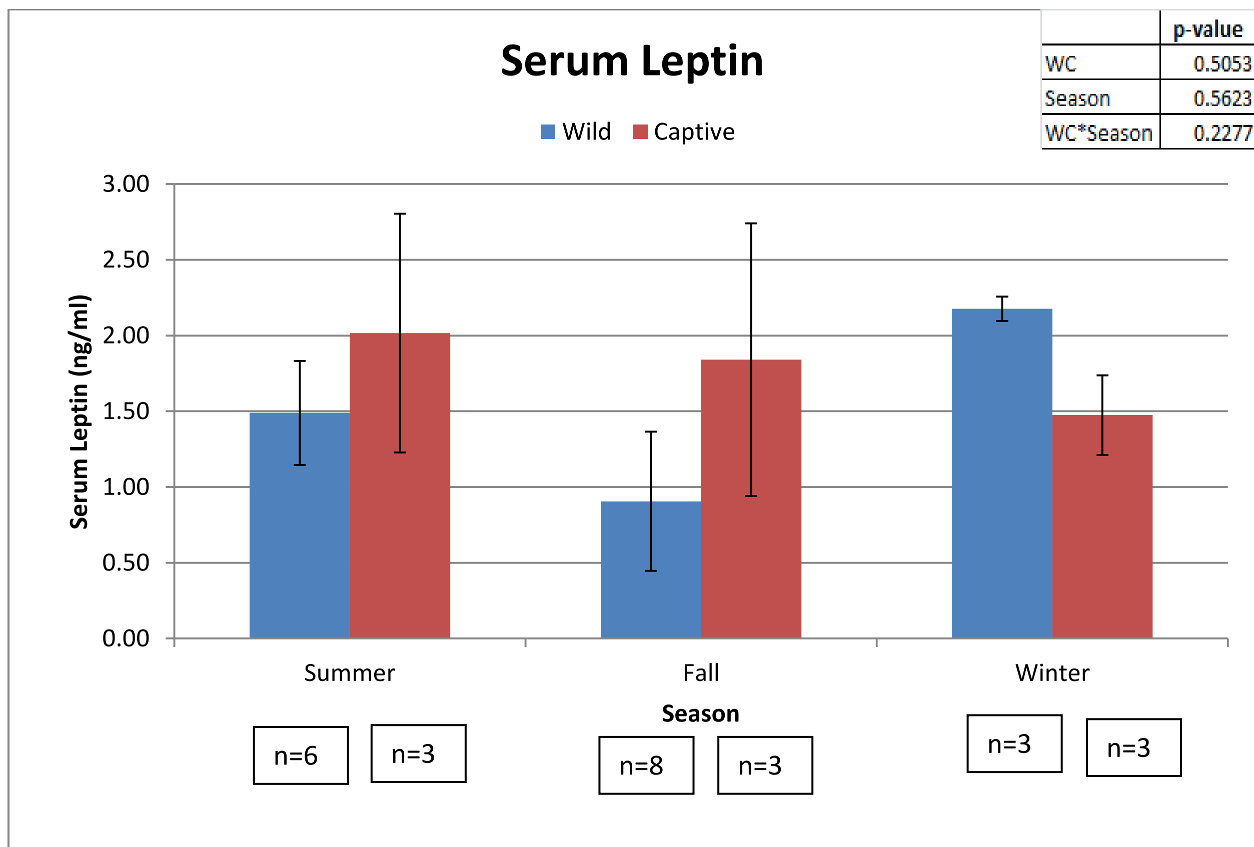

Figure 4-8. Mean (± SEM) American black bear serum leptin measured by RIA. Abbreviation: WC, wild vs. captive.

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Adiponectin

Plasma adiponectin concentrations varied significantly across season (p = 0.0247; Figure 4-9). Wild bears tended to have higher adiponectin levels than captive bears, although this trend was not statistically significant (p = 0.0886). Within the wild group, bears in the fall had greater leptin levels than those in summer and winter, based on Fisher's protected LSD.

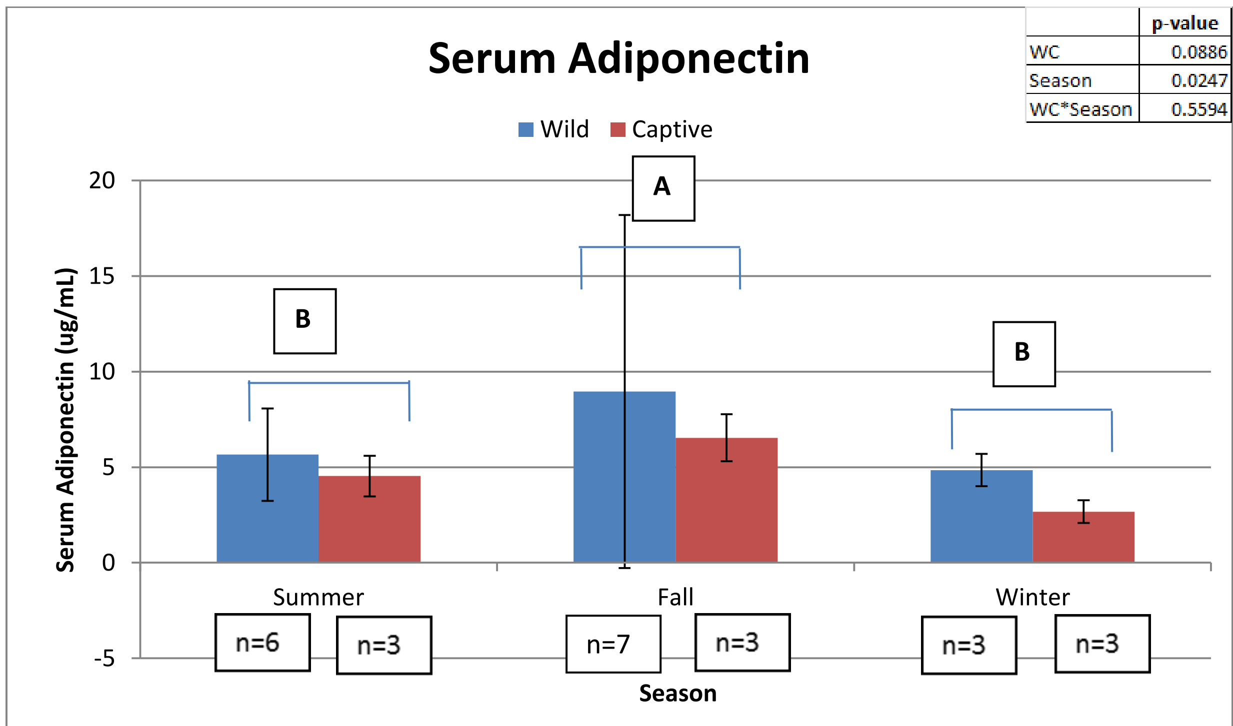

Figure 4-9. Mean (± SEM) American black bear serum adiponectin measured by ELISA. Groups with the same superscripts are not significantly different from each other. Abbreviation: WC, wild vs. captive.

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Adipocyte size

The subcutaneous and abdominal adipose tissues measured were primarily from wild bears, with the exception of one captive winter subcutaneous sample and one captive winter abdominal sample. There were no statistical differences in adipocyte size between subcutaneous and abdominal adipose tissue or amongst seasons (Figure 4-10). Each subcutaneous and abdominal adipose tissue sample measured were from different bears, with the exception of sampling the same captive bear for the winter abdominal sample and for winter subcutaneous adipose tissue.

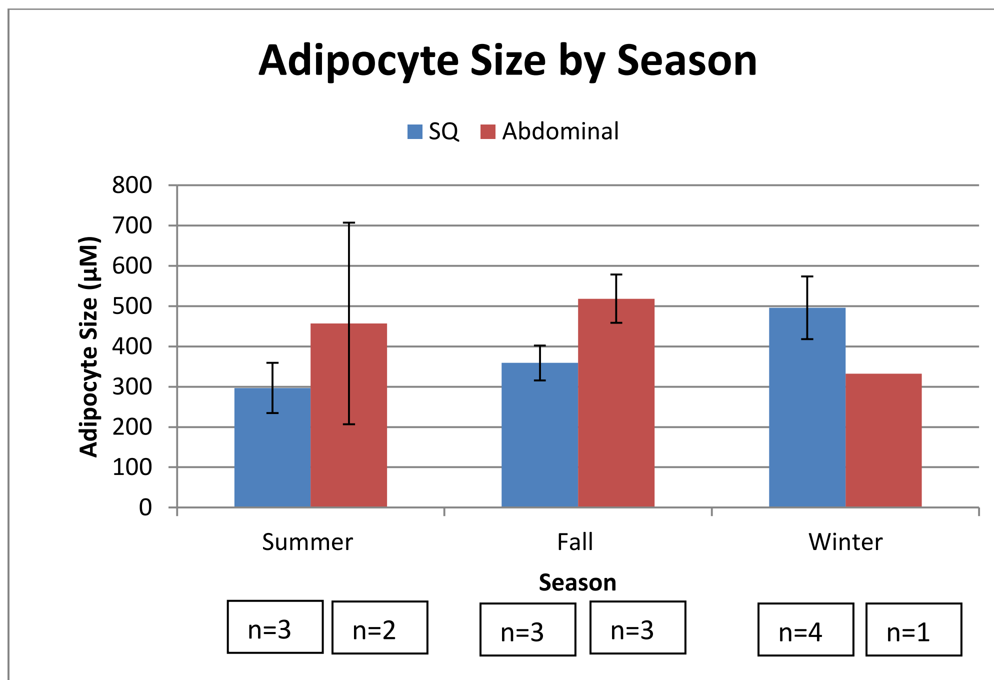

Figure 4-10. Mean (± SEM) American black bear adipocyte size for subcutaneous and abdominal adipose tissue.

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QPCR

Quantitative RT-PCR was used to profile expression of leptin, adiponectin, PDK4 and PRKAA1 in adipose tissue, which were collected from a subset of 24 bears, as a means to evaluate metabolic markers in bear subcutaneous white adipose tissue. Expression levels of PDK4 and PRKAA1 were also determined in liver samples, which were collected from a subset of 8 bears, to compare metabolic effects of season and captivity between the two tissues.

Leptin expression

Similar to the findings for serum leptin, there were no significant differences in leptin expression between wild vs. captive bears or across seasons (Figure 4-11). There was no significant interaction between status and season.

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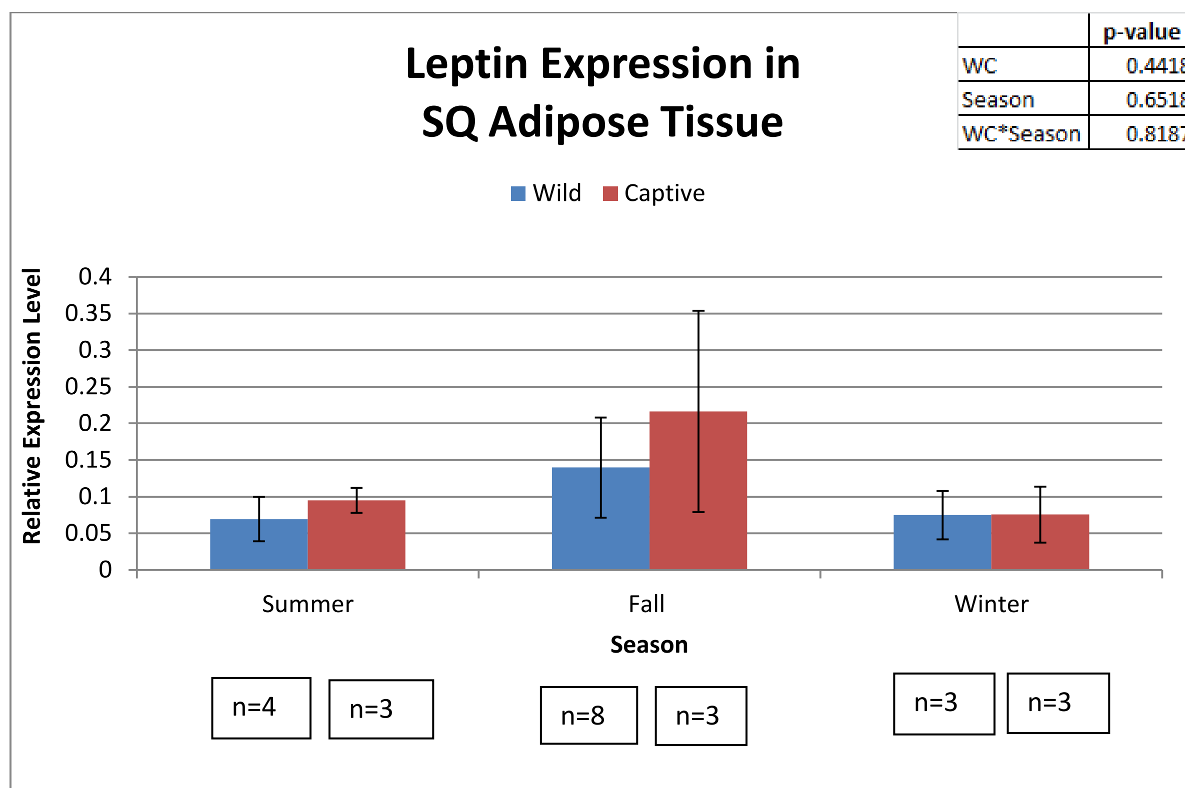

Figure 4-11. Mean (± SEM) American black bear subcutaneous adipose tissue leptin expression determined by QPCR. Abbreviation: WC, wild vs. captive.

Adiponectin expression

There was no significant difference between captive vs. wild bears in any season for adiponectin expression, nor was there a seasonal effect, but status and season interacted significantly with respect to adiponectin expression in subcutaneous adipose tissue ($p = 0.0033$; Figure 4-12). Captive fall bears had a significantly greater expression of adiponectin in subcutaneous adipose tissue than captive summer or captive winter bears ($p < 0.05$).

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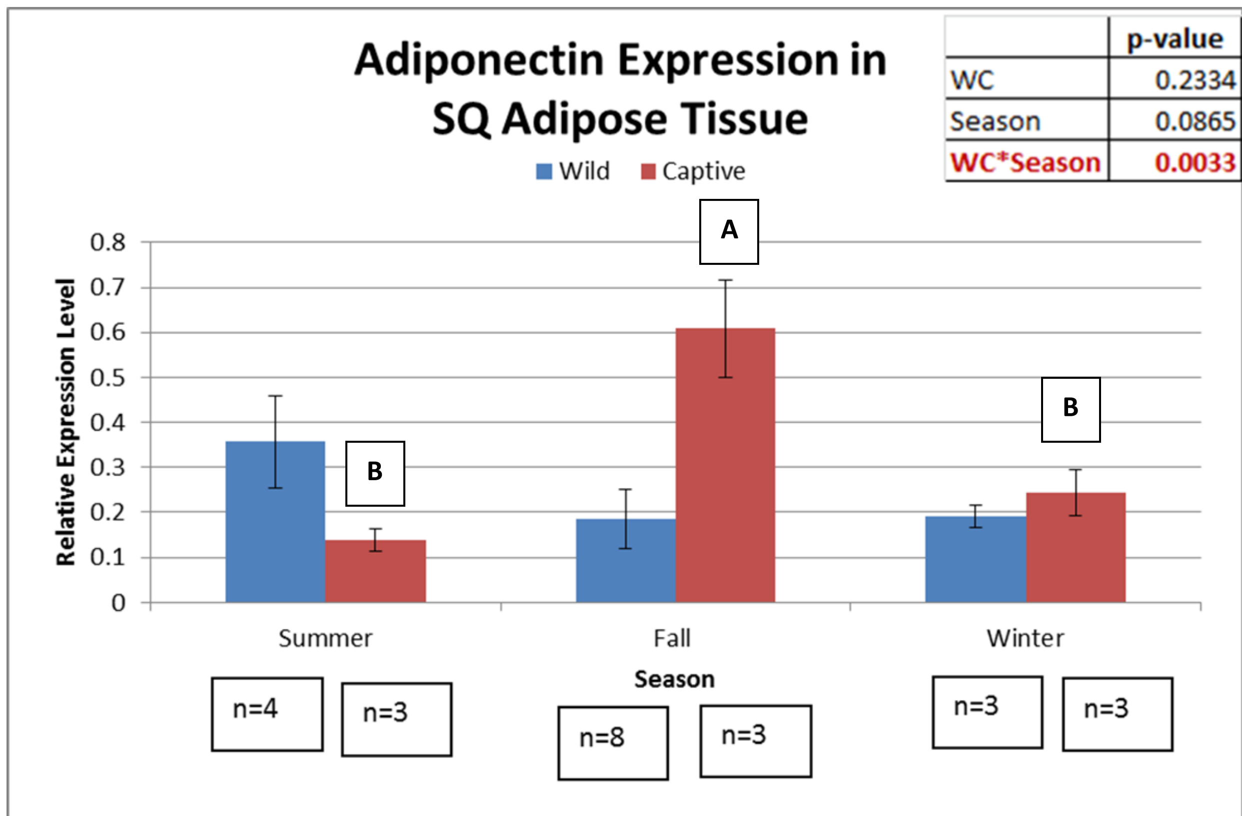

Figure 4-12. Mean (± SEM) American black bear subcutaneous adipose tissue adiponectin expression determined by QPCR. Captive bears with the same superscripts are not significantly different from each other. Abbreviation: WC, wild vs. captive.

PDK4 expression

There were no significant differences between captive and wild bears or amongst seasons for adipose tissue or liver PDK4 expression (Figures 4-13 and 4-14). PDK4 expression tended to be higher in wild vs. captive bears ($p = 0.1080$), especially during summer and fall, but this trend did not reach statistical significance. There was no significant interaction between

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status and season for subcutaneous adipose PDK4 expression. The statistical analysis of liver PDK4 expression was hampered by low numbers of samples.

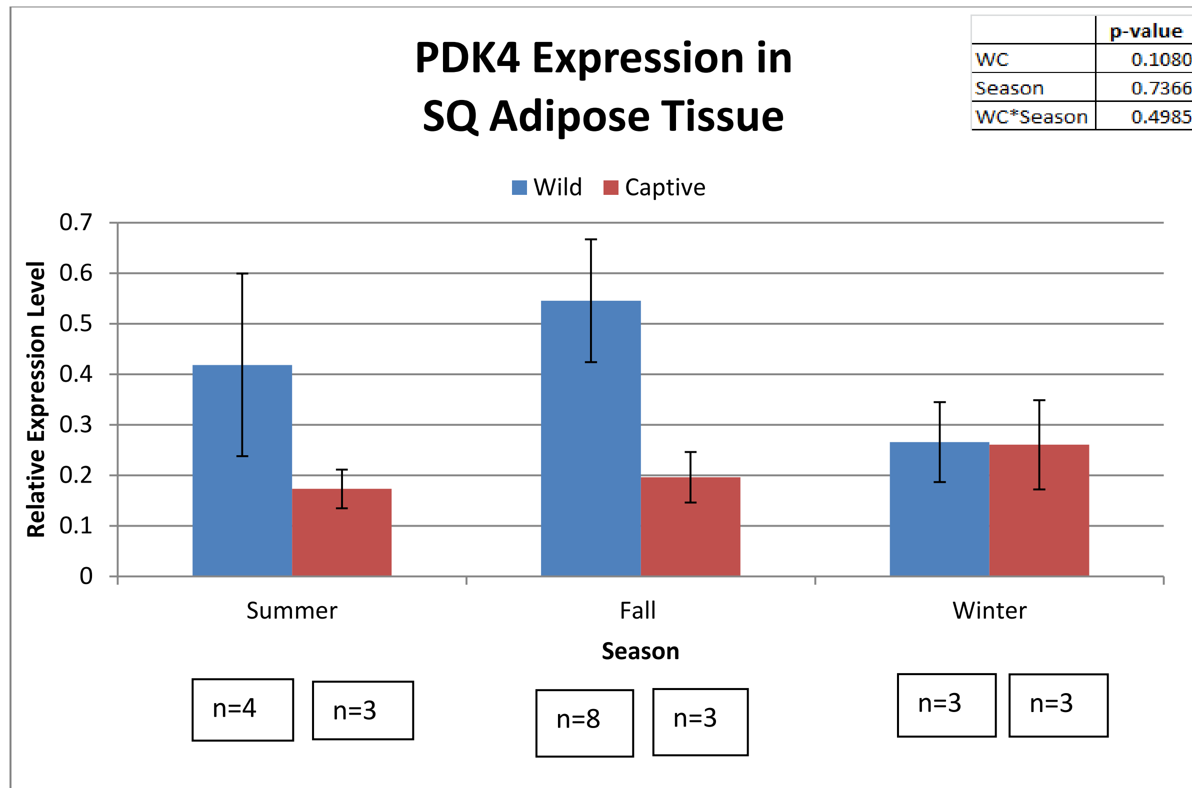

Figure 4-13. Mean (± SEM) American black bear subcutaneous adipose tissue PDK4 expression determined by QPCR. Abbreviation: WC, wild vs. captive.

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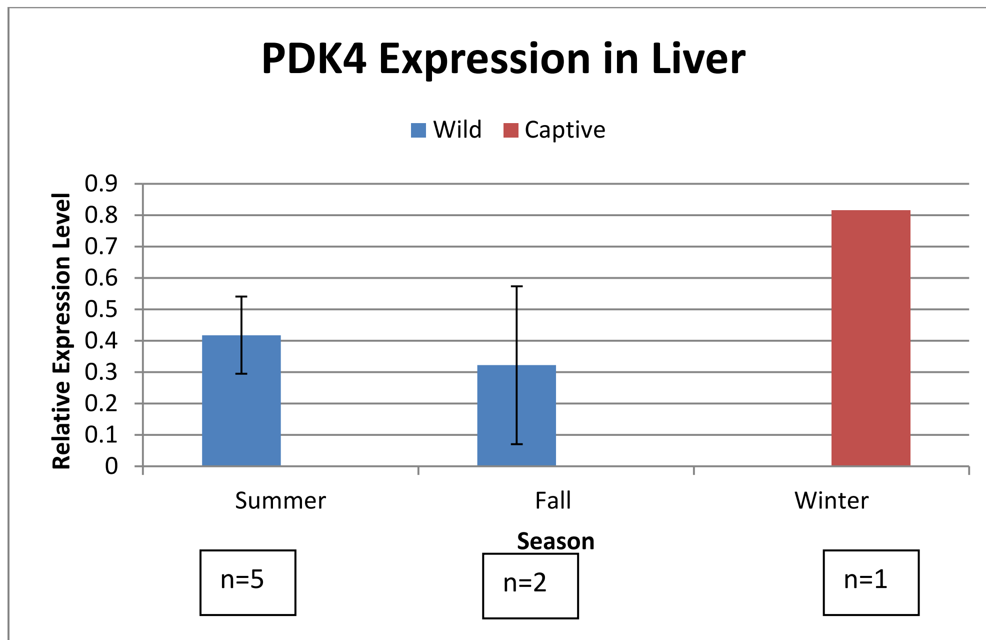

Figure 4-14. Mean (± SEM) American black bear liver PDK4 expression determined by QPCR.

PRKAA1 expression

Expression of PRKAA1 varied significantly with season, with fall bears having greater expression than either summer and winter bears (p = 0.0022). There was also a significant interaction of status and season (p = 0.0267). Captive fall bears had significantly greater SQ adipose tissue PRKAA1 expression than both captive summer bears and captive winter bears (Figure 4-15). There were no significant differences in adipose tissue PRKAA1 expression between captive and wild bears. There were also no significant differences between seasons for liver PRKAA1 expression. PRKAA1 expression was greatest in the liver in the winter, but there was only one winter bear sampled (Figure 4-16).

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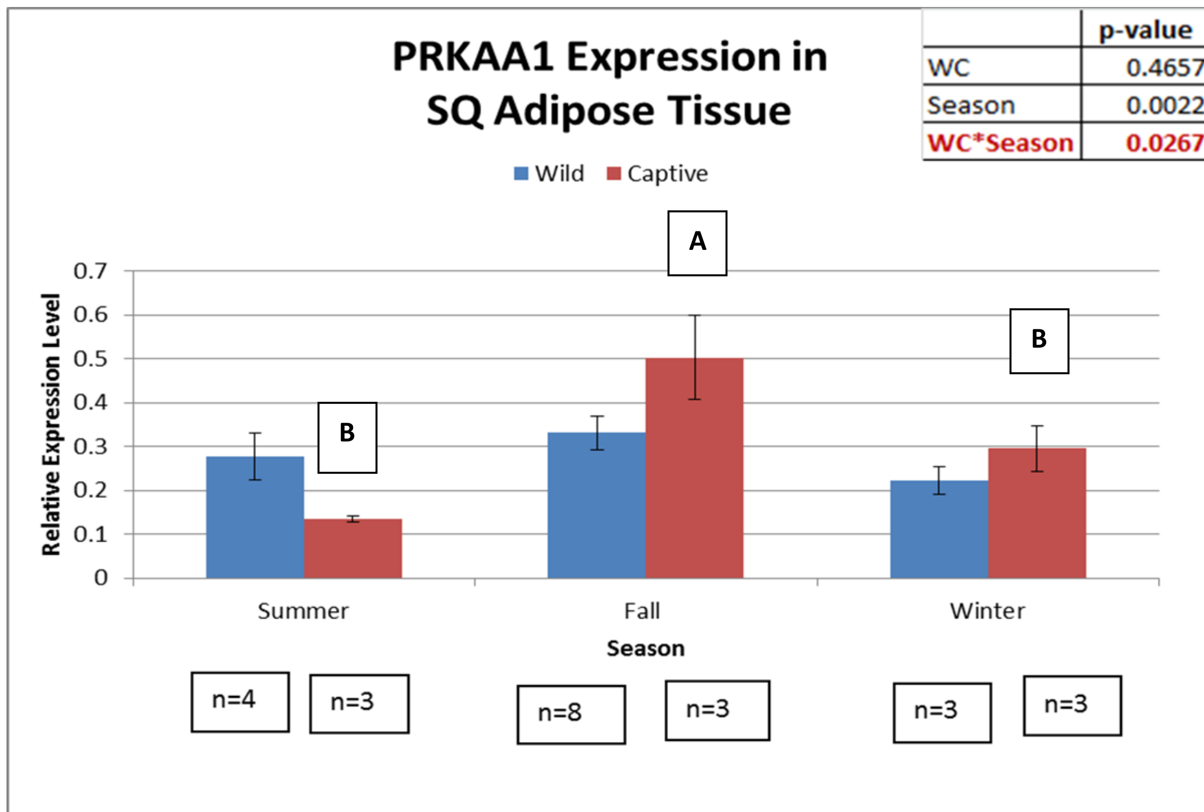

Figure 4-15. Mean (± SEM) American black bear subcutaneous adipose tissue PRKAA1 expression determined by QPCR. Fall bears had significantly greater PRKAA1 expression than summer or winter bears. Captive bears with the same superscripts are not significantly different from each other. Abbreviation: WC, wild vs. captive.

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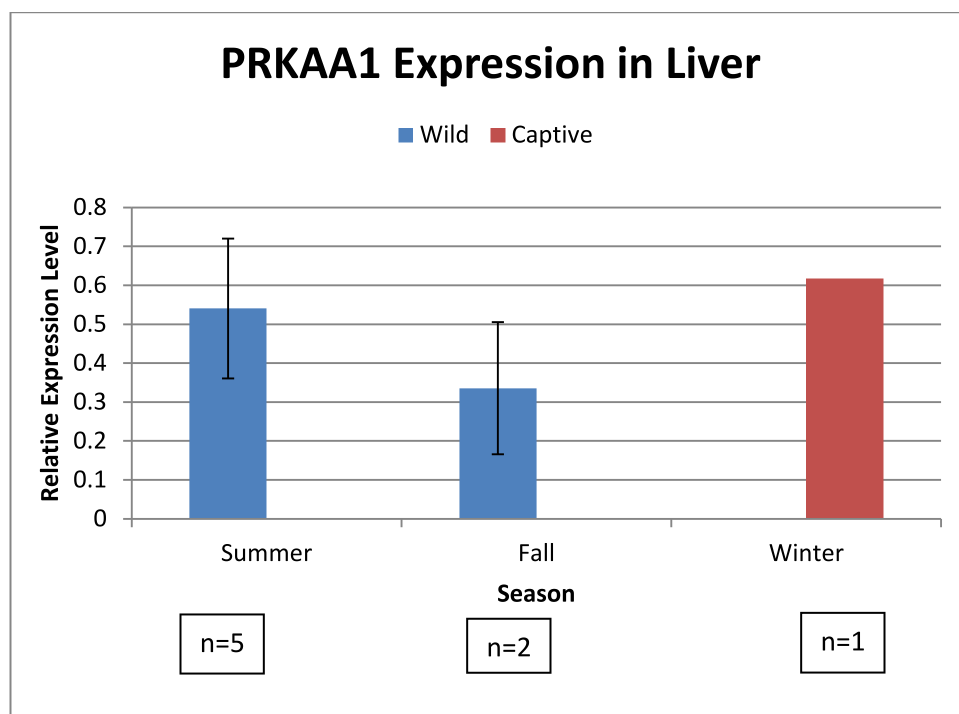

Figure 4-16. Mean (± SEM) American black bear liver PRKAA1 expression determined by QPCR.

Correlation matrix

Correlation analyses across the entire data set was used to identify significant relationships between traits, as a means to identify factors associated with seasonal variation in fatness and other relationships between traits. Pearson's correlation coefficient and the p-value associated with each pairwise correlation are presented in Table 4-1. A correlation matrix was designed to compute the correlation coefficients for each serum/plasma assay and adipokine expressed in subcutaneous adipose tissue that was measured by QPCR. Each top set of data in the matrix represents the correlation coefficient as an r value. P-values were calculated and reported underneath the r values. Significant p-values and their r values are

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highlighted in red. The shaded data represent a negative correlation and unshaded data represent a positive correlation.

PRKAA1 expression and subcutaneous adipose tissue adiponectin expression, and PRKAA1 expression and subcutaneous adipose tissue leptin expression were both significantly and positively correlated ($r = 0.5417$; $p = 0.0063$; Figure 4-17; and $r = 0.4376$; $p = 0.0325$; Figure 4-18, respectively). NEFA was significantly and positively correlated with PDK4 subcutaneous adipose tissue ($r = 0.4727$; $p = 0.0197$; Figure 4-19). Serum leptin was significantly and negatively correlated to subcutaneous adipose tissue PDK4 ($r = -0.5904$; $p = 0.0038$; Figure 4-20). Triglycerides were significantly and positively correlated with beta-hydroxybutyrate ($r = 0.4226$; $p = 0.0397$; Figure 4-21). Serum adiponectin was significantly and negatively correlated with BMI ($r = -0.4569$; $p = 0.0326$; Figure 4-22).

Subcutaneous adipose tissue adipocyte size was correlated negatively with NEFA (Figure 4-23) and positively with SQ leptin expression (Figure 4-24), with r values of -0.5743 and 0.5478, respectively.

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Table 4-1. Correlation matrix for each serum/plasma assay and adipokine expressed in subcutaneous adipose tissue measured by QPCR. Listed at the top of each cell are r values and listed at the bottom of each cell are p-values. Shaded cells represent a negative correlation and unshaded cells represent a positive correlation. Values in red represent a significant p-value below their r value.

	Adiponectin Fat QPCR	Leptin Fat QPCR	PDK4 Fat QPCR	PRKAA1 Fat QPCR	BMI	Beta-hydroxybutyrate	NEFA	Triglycerides	Leptin RIA	Adiponectin ELISA	Adipocyte Size Abdominal	Adipocyte Size SQ
Adiponectin Fat	1.0000											
Leptin Fat QPCR	0.3207											
	0.1265	1.0000										
PDK4 Fat QPCR	0.0584	0.0628										
	0.7865	0.7706	1.0000									
PRKAA1 Fat QPCR	0.5417	0.4376	0.3013									
	0.0063	0.0325	0.1525	1.0000								
BMI	0.2171	0.1106	0.4204	0.0653								
	0.3318	0.6241	0.0634	0.7729	1.0000							
Beta-hydroxybutyrate	0.1829	0.2436	0.1160	0.0306	0.3837							
	0.3922	0.2515	0.5893	0.8872	0.0779	1.0000						
NEFA	0.3580	0.2208	0.4727	0.0357	0.0750	0.1487						
	0.0859	0.2999	0.0197	0.8683	0.7399	0.4880	1.0000					
Triglycerides	0.0425	0.0390	0.3721	0.0313	0.3780	0.4226	0.0383					
	0.8435	0.8563	0.0734	0.8845	0.0828	0.0397	0.8591	1.0000				
Leptin RIA	0.0859	0.3691	0.5904	0.0173	0.2340	0.1695	0.3424	0.3105				
	0.7040	0.0909	0.0038	0.9389	0.2947	0.4508	0.1188	0.1596	1.0000			
Adiponectin ELISA	0.1266	0.1141	0.0735	0.1634	0.4569	0.0335	0.1882	0.0632	0.0531			
	0.5556	0.5956	0.7328	0.4454	0.0326	0.8767	0.3784	0.7692	0.8145	1.0000		
Adipocyte Size Abdominal	0.0955	0.3473	0.5665	0.5170	0.1611	0.6979	0.4652	0.6632	0.8288	0.2655		
	0.8572	0.4999	0.2411	0.2936	0.7958	0.1231	0.3526	0.1511	0.0828	0.6111	1.0000	
Adipocyte Size SQ	0.0949	0.5487	0.0254	0.2366	0.0805	0.3148	0.3382	0.3821	0.4123	0.1896	0.6542	
	0.7941	0.1004	0.9445	0.5103	0.8369	0.3755	0.3390	0.2758	0.2701	0.5998	0.2310	1.0000

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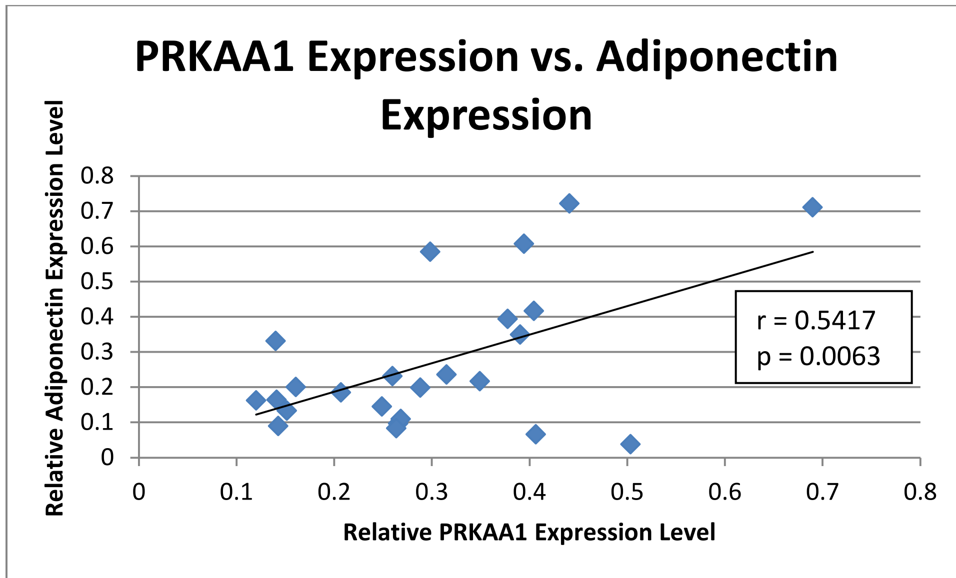

Figure 4-17. American black bear relative PRKAA1 expression levels plotted against relative adiponectin expression levels.

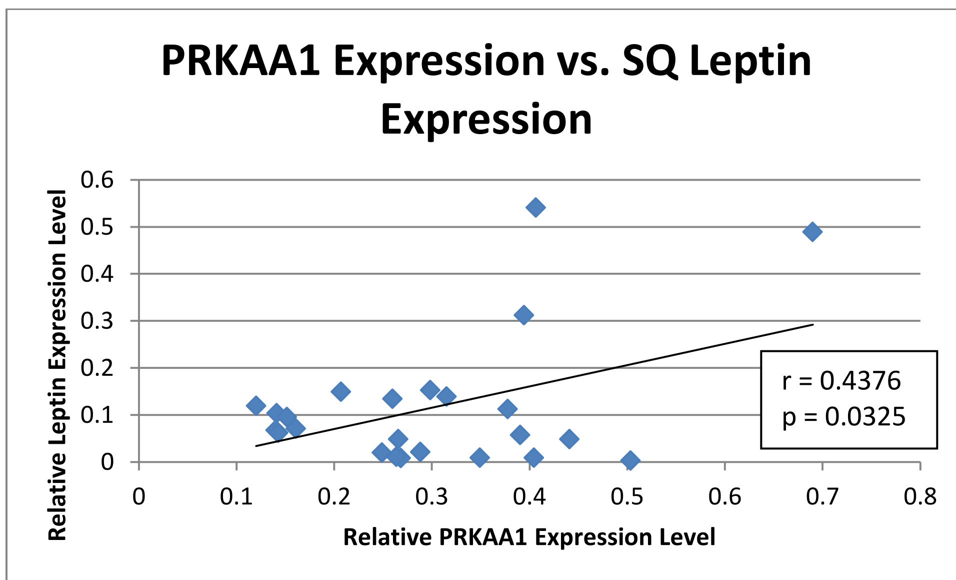

Figure 4-18. American black bear relative PRKAA1 expression levels plotted against relative subcutaneous adipose leptin expression levels.

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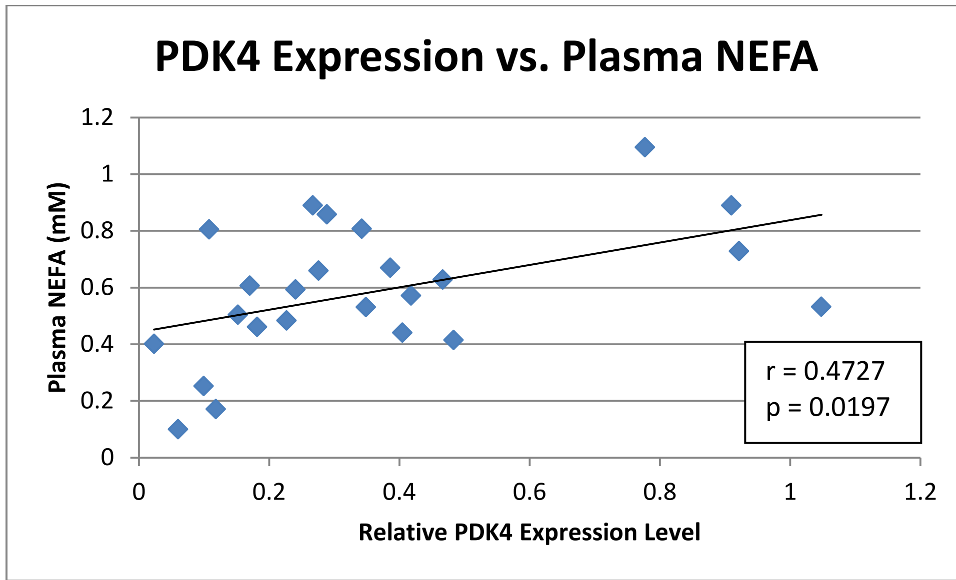

Figure 4-19. American black bear relative subcutaneous adipose PDK4 expression levels plotted against plasma NEFA concentrations.

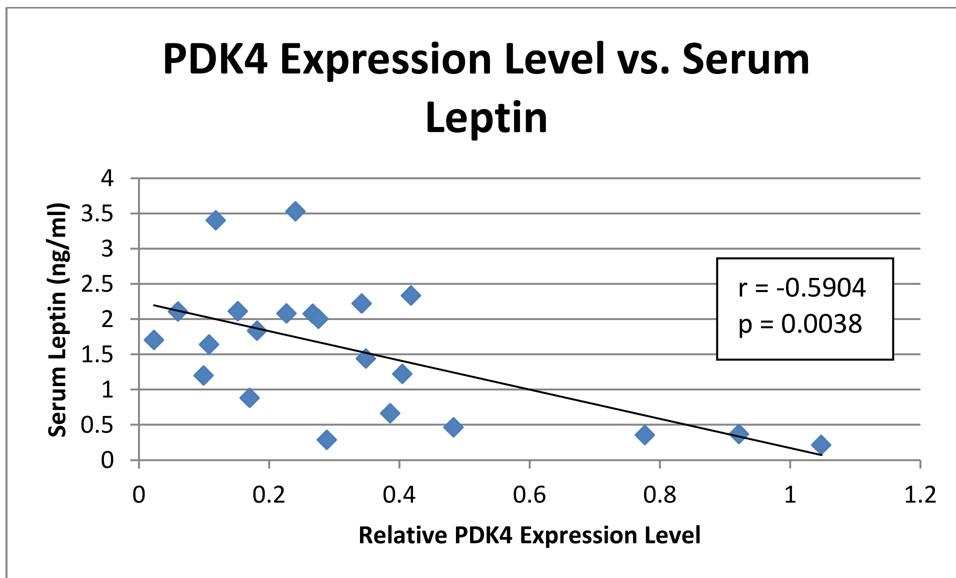

Figure 4-20. American black bear relative subcutaneous adipose PDK4 expression levels plotted against serum leptin concentrations.

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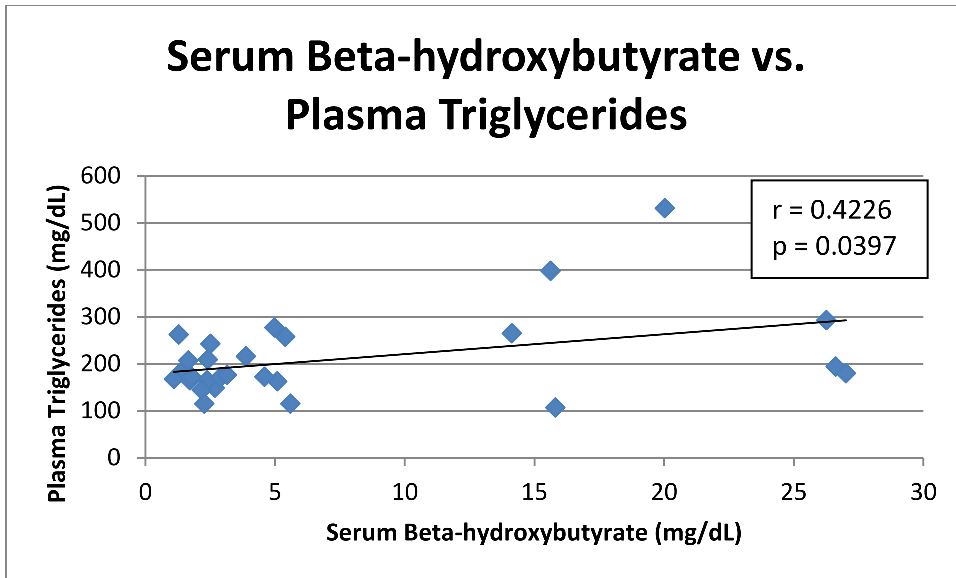

Figure 4-21. American black bear serum beta-hydroxybutyrate concentrations plotted against plasma triglyceride concentrations.

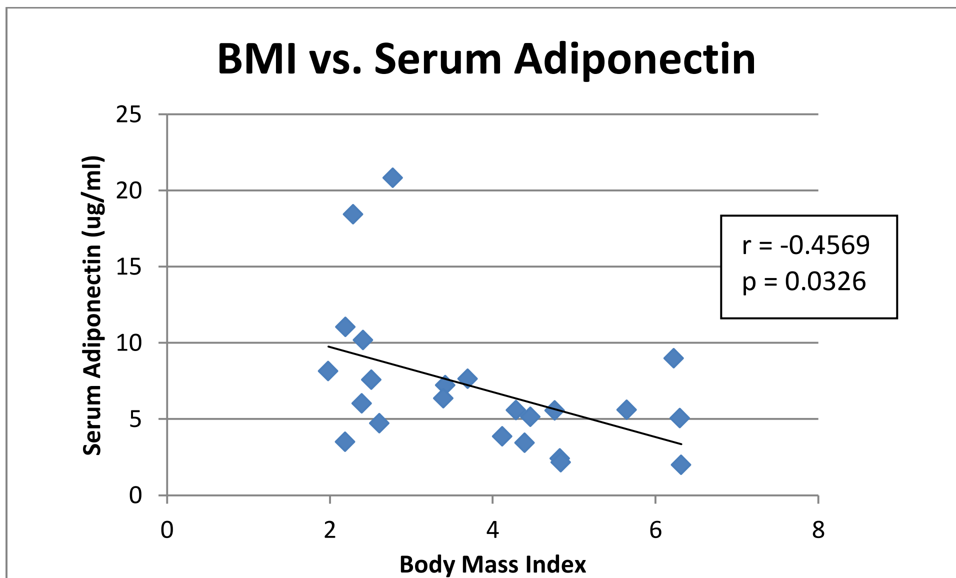

Figure 4-22. American black bear body mass index (BMI) plotted against serum adiponectin concentrations.

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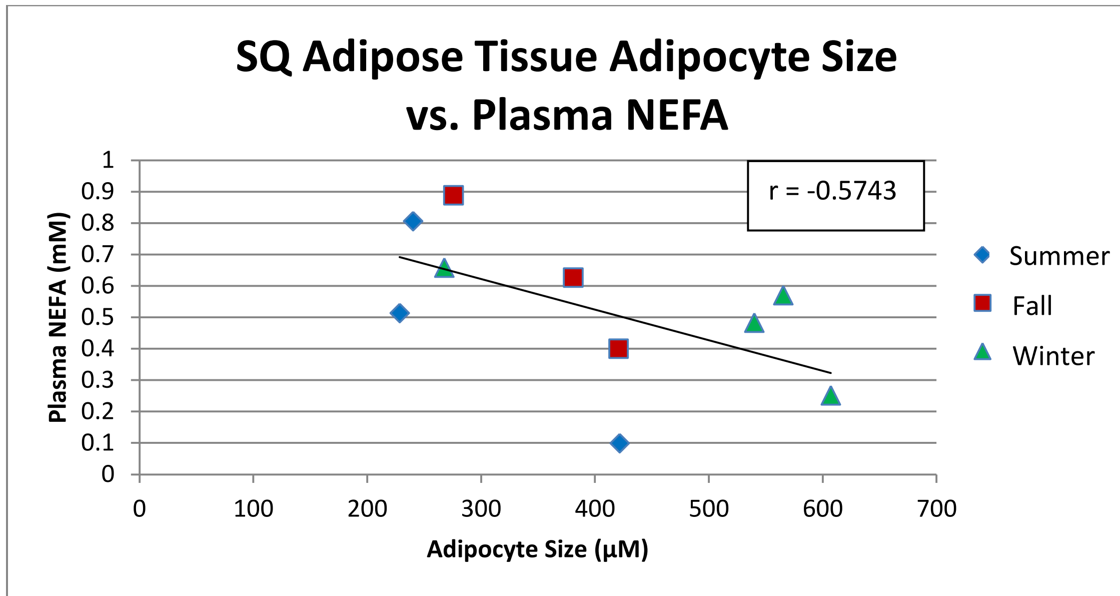

Figure 4-23. Mean American black bear subcutaneous adipose tissue adipocyte size vs. plasma non-esterified fatty acids measured by assay.

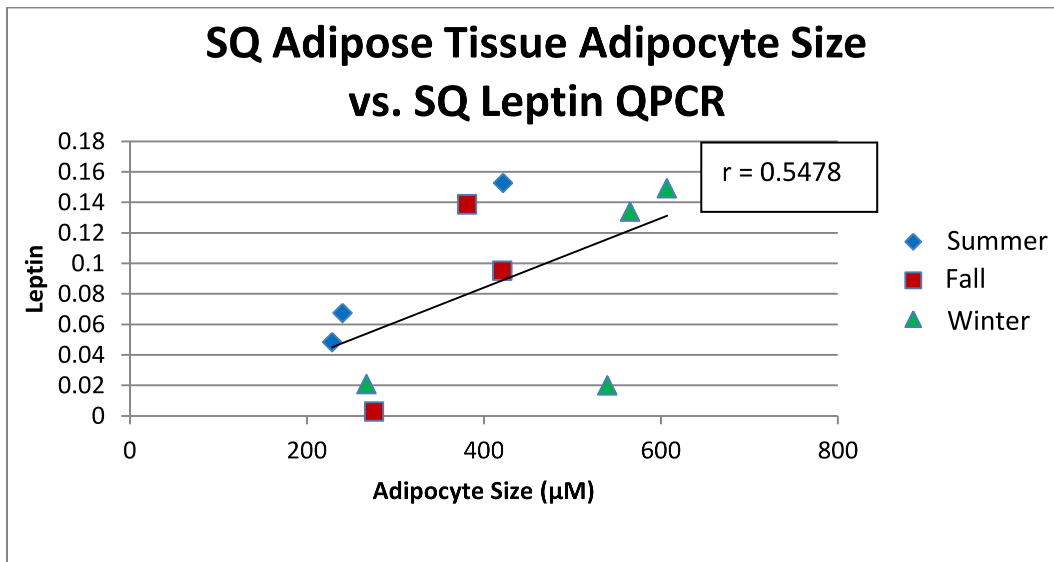

Figure 4-24. Mean American black bear subcutaneous adipose tissue adipocyte size vs. subcutaneous adipose tissue leptin expression determined by QPCR.

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DISCUSSION

Metabolic measures

Lipid metabolism

Plasma triglycerides were measured as an index of circulating lipid levels. Triglycerides are an indicator of food and energy abundance, and it was expected for captive bears with high BMI values and possibly fall bears, which should have been depositing fat, to have increased triglyceride concentrations. In contrast, we did not see any differences in triglyceride levels amongst groups or seasons.

Plasma NEFA was measured as an index of lipolysis. It was expected for winter bears to have the highest NEFA levels since they were hibernating, not eating, and depending on their fat for energy. While no significant differences were observed between groups or amongst seasons, surprisingly, wild fall bears' plasma NEFA raw values were greatest of all groups. Contributing to this was the wild fall bears from the GSMNP experiencing poor forage conditions, and the wild fall bears in New Jersey being mostly lean juveniles. Another confounding factor was that all the wild winter bears were lactating females, which may have altered their lipid metabolism.

Serum beta-hydroxybutyrate was measured as an indicator of fatty acid oxidation. Fatty acid oxidation was significantly less in captive bears than in wild bears, in each season, which is consistent with our observed differences in BMI between the two study groups. This difference likely results from differences in the availability of food and activity. Beta-hydroxybutyrate

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concentrations were expected to be greatest during the winter, due to fat metabolism occurring during hibernation. There was a tendency for wild bears to have higher levels during winter, although this did not reach statistical significance. In contrast, there was very little seasonal variation in fatty acid oxidation in captive bears, based on this serum marker.

Adipokines

We chose two adipokines, leptin and adiponectin, to measure in the bears. Leptin regulates food intake and energy expenditure, and adiponectin regulates glucose levels and fatty acid oxidation. In contrast to the finding of Tsubota (Tsubota et al., 2008), serum leptin levels in our bears did not change over season. Fall bears were expected to have greater serum leptin concentrations than summer bears. One possible explanation for our failure to discern a change is that other molecules may have reacted with the antibody in the RIA kit. A study of leptin in the feral raccoon (*Procyon lotor*) found the multispecies RIA assay to be less effective in measuring concentrations than a canine-leptin-specific ELISA (Shibata et al., 2005). RIA leptin concentrations were consistently much lower than ELISA values, and the results showed clear seasonal variations in leptin levels determined by the ELISA. We ran a number of bear serum samples on a canine-leptin-specific ELISA, but were unable to detect leptin in those samples (unpublished data). Our lack of seasonal serum leptin changes are confirmed, however, by our failure to find differences between groups or amongst seasons for subcutaneous adipose tissue leptin expression. Captive and wild bears did tend to have more SQ adipose tissue leptin

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expression in the fall as compared to summer and winter. In mice, an increase in leptin results in a decrease in food intake (Friedman, 2011), but in the bear leptin seems to have the opposite effect, or possibly fall bears become leptin resistant.

In contrast to leptin, serum adiponectin was significantly greater during the fall for both wild and captive bears, as compared to the summer and winter bears. This was surprising because the captive fall bears had the greatest BMI values, and adiponectin is normally highest when animals are lean. Subcutaneous adipose adiponectin expression followed a similar pattern as serum adiponectin in captive bears, with significantly greater expression in captive fall bears, as compared to captive summer and captive winter bears. It was expected for winter bears to have the greatest amounts of adiponectin, due to oxidation of fatty acids during hibernation. Wild winter bears consisted of hibernating lactating females, all of which had larger body masses than the wild summer bears, and the wild winter bears did tend to have lower adiponectin concentrations than the wild summer bears.

Molecular markers of metabolism

We measured PDK4 and PRKAA1 as molecular markers of metabolism because both function as molecular energy sensors that alter cellular metabolism in response to changes in energy abundance. These genes may be involved in regulating the metabolic fuel switch from carbohydrate oxidation during pre-hibernation to fatty acid oxidation during hibernation. We expected PDK4 and PRKAA1 expression to be highest during the winter.

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PDK4 expression in SQ adipose tissue showed a trend toward higher expression in wild vs. captive bears, but this difference was not statistically significant. In captive bears, PDK4 expression varied very little between seasons, which was expected, since these bears had access to food daily. In wild bears, PDK4 expression varied more dramatically amongst seasons, most likely due to the food scarcity in the GSMNP during the summer and fall, and that the wild winter bears were lactating females that were hibernating. Three of the fall New Jersey wild bears were less than two years old, and were small and lean, but their size was appropriate for their age. It was expected for both wild and captive bears to have the greatest expression of PDK4 during the winter when they were hibernating, since they were not eating and should have been oxidizing fatty acids for energy. Although there were no seasonal differences in PDK4, serum beta-hydroxybutyrate concentrations were slightly, but not significantly, higher in wild winter bears, suggesting those bears were oxidizing fatty acids during hibernation. Our results contrast with a study in 13-lined ground squirrels that found that low PDK4 expression, likely inhibited by high serum insulin concentrations during the fall, and PDK4 expression were up-regulated in the heart, skeletal muscle and white adipose tissue during hibernation (Buck et al., 2002).

PRKAA1 expression was significantly higher in fall bears, in general, and in captive fall bears, as compared to captive summer bears and captive winter bears. It was expected for winter PRKAA1 expression to be greater since fasting stimulates phosphorylation of AMPK. We did only evaluate one component of the regulation and this may partially explain our results. During hibernation of 13-lined ground squirrels, AMPK expression was significantly higher in

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white adipose tissue than in the liver (Horman et al., 2005). Winter fasted euthermic golden-mantled ground squirrels had significantly less AMPK than torpid ground squirrels (Healy et al., 2011). These studies are the opposite of what was found in the bear. Adipose tissue and liver samples were obtained from winter bears towards the end of hibernation, therefore AMPK may be a vital energy sensor at different times during hibernation.

Relationships among traits

The rationale behind correlation analyses was to identify traits that were correlated with fatness, and thus potentially causally related to the fat loss that occurs during hibernation. PRKAA1 expression was positively correlated to adiponectin and leptin expression in subcutaneous adipose tissue. The relationship of PRKAA1 with adiponectin was expected because both activate fatty acid oxidation pathways (Yoon et al., 2006). A significant, positive correlation between leptin and PRKAA1 expression may arise from the fact that leptin signaling has been shown to be 'upstream' of AMPK in at least some tissues, in which activation of the leptin receptor leads to AMPK phosphorylation (Bjorbaek and Kahn, 2004). Although PDK4 expression did not differ significantly across seasons or captive status, expression levels were significantly and positively correlated to plasma NEFA, and inversely to plasma leptin concentration, and approached a significant negative correlation with BMI (significance = 0.0634). These relationships are consistent with the expected role of PDK4 as a fuel switch that increases fatty acid oxidation during times of food scarcity, and with the concept that PDK4 is

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associated with fat loss, while plasma leptin should reflect increased fatness as occurs during preparation for hibernation. BMI was negatively correlated to adiponectin measured by ELISA. These results were expected because as BMI increases, adiponectin decreases, since adiponectin is known to be low in obesity (Kern et al., 2003). Beta-hydroxybutyrate was positively correlated to triglycerides, which is expected because high beta-hydroxybutyrate is associated with fatty acid oxidation and triglycerides are made up of fatty acids.

Adipocyte size was measured as an additional index of adiposity. Although there were no statistical differences between seasons or subcutaneous vs. abdominal, the wild bears sampled in the summer and fall were lean, therefore the smaller adipocyte size was expected. The winter subcutaneous tissue samples consisted of two lactating females in New Jersey and two fattier captive bears. The winter abdominal tissue sample was from only one captive bear. If the winter subcutaneous tissue samples had instead been from wild bears in the GSMNP, it would have been expected for their adipocyte size to be smaller than summer and fall, due to fat loss during hibernation.

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CHAPTER V

FATTY ACID ANALYSIS

We performed a pilot study to characterize the fatty acid profiles of black bear adipose tissue and to identify potential seasonal differences that could indicate seasonal changes in fatty acid metabolism. Fatty acids were measured in subcutaneous and abdominal adipose tissue in wild and captive bears for the summer, fall, and winter seasons (Table 5-1). Summer and fall tissue samples contained two captive bear subcutaneous tissue samples, one wild bear subcutaneous tissue sample, and one wild bear abdominal tissue sample. The winter tissue samples contained three captive bear subcutaneous tissue samples and one captive bear abdominal tissue sample. The tissue samples ranged from 26 to 54 mg in weight.

The fatty acid analysis was performed using gas chromatography – mass spectrometry (GCMS). In the first part of the analysis, lipids were extracted. Each sample had 2 ml of chloroform and 1 ml of methanol added to a glass homogenizer, < 1 g of tissue added, and was homogenized for 1 minute. The homogenate was poured into teflon-capped tubes and centrifuged at 3000 g for 10 minutes. The supernatant was added to a new glass tube, 0.2 x volume (0.75 ml) was added, and centrifuged at 3000 g for 20 minutes. The upper layer was then discarded.

The second part of the analysis was for acid-catalyzed fatty acid methylation. The solvent from the lipid samples was evaporated by directing a stream of nitrogen gas into the tubes until all of the liquid was evaporated. Each tube had 1 ml of 0.3M KOH in 90% methanol

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added and heated at 80 °C for 60 minutes to hydrolyze the lipids, releasing the free fatty acids. Afterward, 0.33 ml of chloroform, 0.33 ml of methanol, and 0.33 ml of 14% boron trifluoride (BF3) were added to each sample, and heated at 90 °C for 90 minutes. BF3 is an acid which catalyzes the transfer of a methyl group (CH_3) from methanol to the carboxyl end of the fatty acids. Each sample had 0.55 ml of hexane and 0.275 ml of water added, was vortexed for 1 minute, and then placed on the bench top to form two layers. The upper layer contained mostly hexane, which contains the fatty acids (fatty acid methyl esters). The upper hexane layer was transferred to auto-sampler vials, and the samples were evaporated using nitrogen gas. Hexane containing nonadecanoic acid (odd-chain fatty acid which served as the internal standard for estimating instrument variability) was reconstituted.

Three major fatty acids were identified: cis-11-octadecenoic acid (also known as cis-vaccenic acid), palmitic acid, and cis-7-hexadecenoic acid (Table 5-1). Palmitic acid concentration was highest in the summer bears. In the European brown bear (*Ursus arctos arctos*), palmitic acid in plasma was increased in the winter, while stearic acid was decreased in the winter (Hissa et al., 1998).

Figures 5-1 to 5-5 depict seasonal changes of the major fatty acids found in bear tissues. Figures 5-3, 5-4, and 5-5 show changes in the ratio of the fatty acids rather than an individual fatty acid. Generally, fatty acid desaturation increases in the fall and winter. In all but one of these fatty acids, desaturation is increased in the fall and winter. Eicosapentaenoic acid (EPA) concentrations were greatest in the winter. In the European brown bear, EPA in plasma was very low in the winter, while docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA)

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concentrations were very increased (Hissa et al., 1998). In wild and captive polar bears (*U. maritimus*), fatty acid composition of triacylglycerols in adipose tissue were different due to the difference in diets (Colby et al., 1993).

During hibernation, metabolic energy is mostly obtained from lipids that are stored in the form of triglycerides in white adipose tissue (Dark, 2005). Fatty acids vary by diet. Mammals are able to synthesize saturated fatty acids and monounsaturated acids, but they are not able to synthesize polyunsaturated fatty acids (PUFAs) (Dark, 2005). Triglycerides in white adipose tissue are needed to preserve fluidity, otherwise at low temperatures lipolysis and energy mobilization from stored lipids would be affected negatively (Frank and Storey, 1995).

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Table 5-1. Fatty acids in American black bears. Fatty acids were measured in µg/ml (same as ppm). Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

			Myristic	Myristoleic	Palmitic	Palmitoleic	Cis-7-hexadecenoic	Stearic	Cis-vaccenic	Oleic	EPA	Arachidonic	DHA
Bear	**Season**	**Total**	**14:00**	**14:01**	**16:00**	**16:01**	**16:01**	**18:00**	**18:01**	**18:01**	**20:05**	**20:04**	**20:06**
Wild Abd	Summer	2126.03	29.96	6.16	609.33	10.3	68.67	141.12	1195.05	52.75	7.54	2.88	2.25
Wild SQ	Summer	1383.19	58.39	9.83	521.25	4.43	66.1	122.72	571.34	21.68	4.17	1.52	1.76
Captive SQ	Summer	723.71	17.01	3.61	317.93	11.53	55.27	32.14	250.02	27.95	5.27	1.28	1.7
Captive SQ	Summer	2267.54	42.17	11.36	786.46	10.62	191.82	96.81	1052.52	48.86	21.19	3.02	2.72
Wild Abd	Fall	1837.95	22.93	4.2	390.2	8.58	43.52	84.32	1237.21	36.46	5.69	1.87	2.97
Wild SQ	Fall	694.75	10.73	2.53	139.97	3.61	19.85	31.5	461.64	17.24	4.66	1.36	1.66
Captive SQ	Fall	1194.98	26.36	16.21	428.96	4.2	162.63	24.89	462.87	53.23	7.77	2.3	5.55
Captive SQ	Fall	1195.54	25.07	15.78	347.16	7.11	178.58	21	531.47	46.7	12.93	2.81	6.94
Captive Abd	Winter	1378.48	22.41	7.63	434.06	9.23	101.87	63.75	678.38	42.64	14.61	1.53	2.39
Captive SQ	Winter	1510.2	22.06	6.94	451.12	10.44	102.72	67.6	786.49	48.16	10.96	1.61	2.1
Captive SQ	Winter	837.99	18.3	7.45	282.59	5.47	91.63	26.63	353.49	28.48	13.65	5.13	5.15
Captive SQ	Winter	1390.26	29.06	11.51	381.17	10.38	146.72	54.52	654.47	49.13	22.99	8.54	21.79

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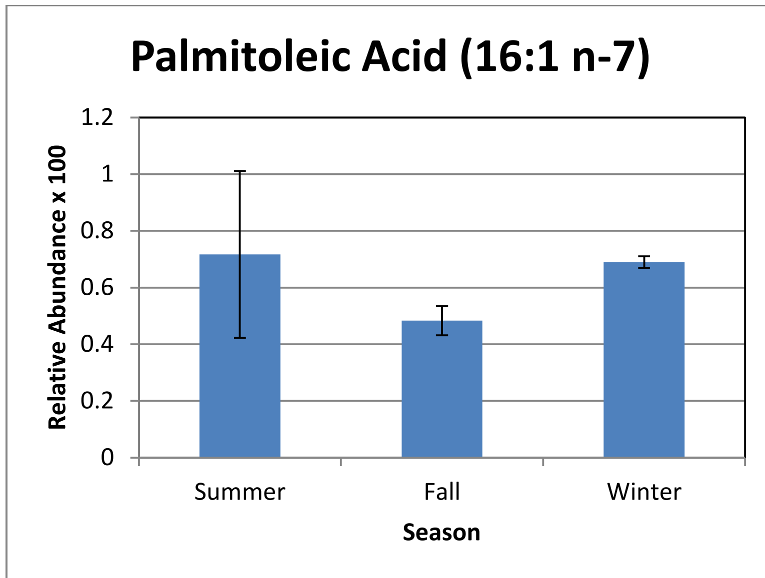

Figure 5-1. Mean (± SEM) relative abundance of American black bear palmitoleic acid by season.

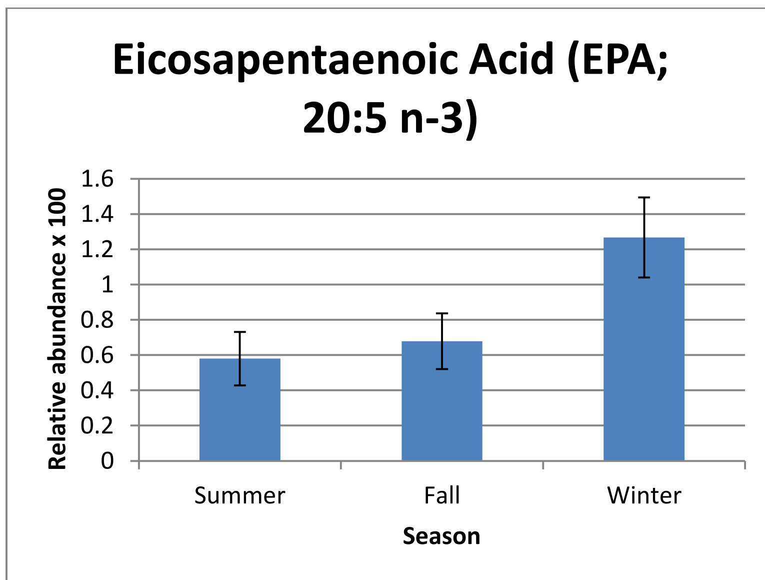

Figure 5-2. Mean (± SEM) relative abundance of American black bear eicosapentaenoic acid by season.

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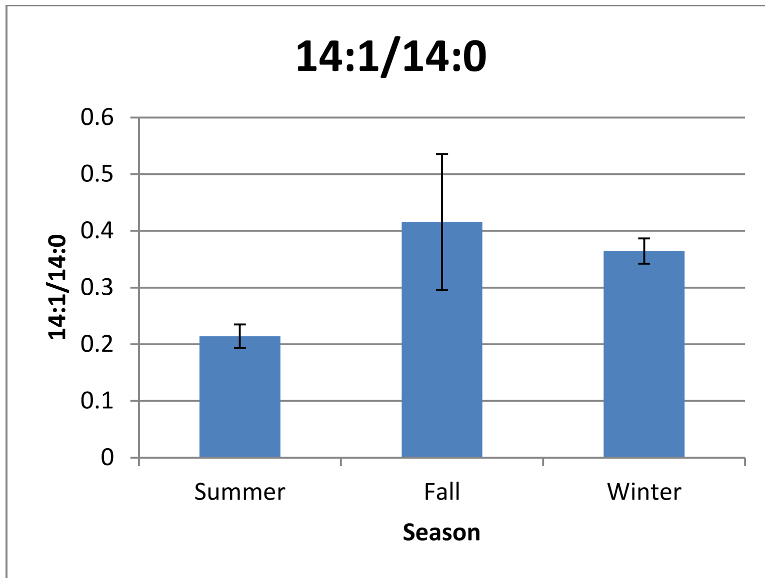

Figure 5-3. Mean (± SEM) ratio of American black bear myristoleic acid (14:1) to myristic acid (14:0).

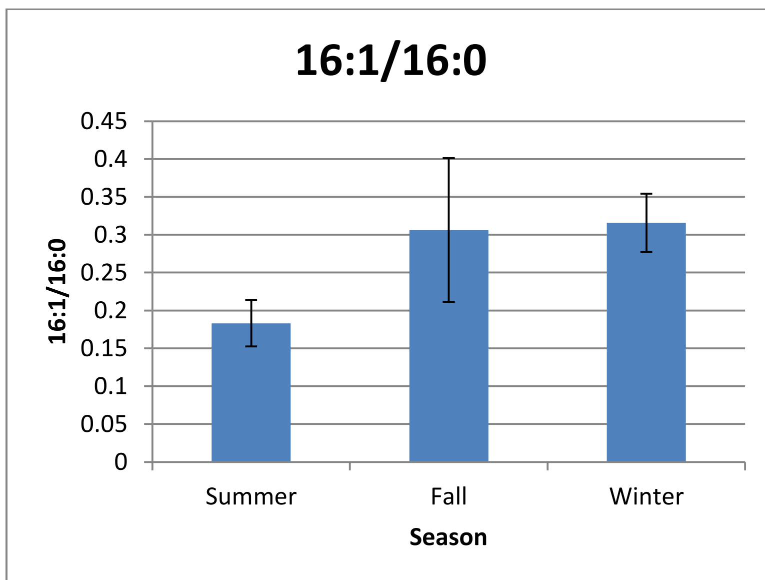

Figure 5-4. Mean (± SEM) ratio of American black bear palmitoleic acid (16:1) and cis-7-hexadecenoic acid (16:1) to palmitic acid (16:0).

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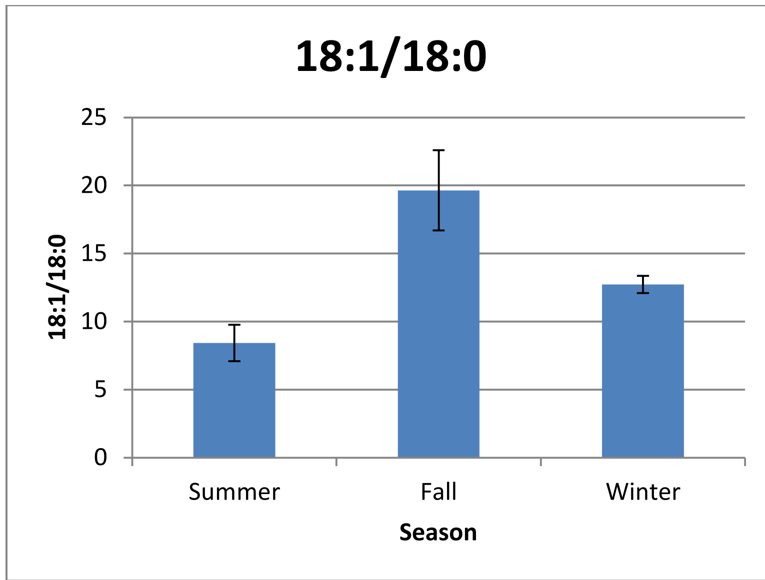

Figure 5-5. Mean (± SEM) ratio of American black bear cis-11-octadecenoic acid (cis-vaccenic; 18:1) and oleic acid (18:1) to stearic acid (18:0).

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CHAPTER VI

FUTURE STUDIES

There were several limitations to the present study. We were not able to sample the same bear repeatedly, thus we were unable to follow the adipokines and markers through individual bear body mass changes. The food scarcity in the GSMNP and the fact that we had only euthanized lean bears for all of the wild summer bears and half of the wild fall bears, impacted many of the results. In repeating this study in the future, it would be advantageous to sample the same bears for each season, by using a tracking collar in the wild or using only captive bears. An issue with the latter is finding a zoo willing to anesthetize their bears several times during the year. Several zoos and wildlife facilities were contacted to participate in this study, and some declined participation even though we had asked for only one sample from each bear. Another issue is funding for a study to use radio-collared bears. The equipment is expensive and expert personnel are required in locating the bear in the wild. Numerous published studies have kept wild bears in a large enclosure to use for obtaining repeated samples. This also would be costly due to the large space needed for the bears, the largest hibernators, in contrast to bats, ground squirrels, and marmots, which can be housed in a laboratory. Also, bears are large and dangerous, and you cannot handle a bear the way you could with a small hibernator, which makes bear studies more challenging.

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