Assessment of Physiological Challenges in Overwintering American Black Bears (*Ursus americanus*): Active Gestation, Neonatal Growth, and Skeletal Muscle Conservation

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

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Assessment of Physiological Challenges in Overwintering American Black Bears (*Ursus americanus*): Active Gestation, Neonatal Growth, and Skeletal Muscle Conservation.

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Abstract (Academic)

The American black bear (ABB) (*Ursus americanus*) exhibits physiological strategies highly synchronized with the environment. Such strategies enable bears to exploit food resources when available and survive the winter months by hibernating without ingesting food or water. However, there are multiple aspects of ABB hibernation physiology that remain unknown. For instance, there is conflicting evidence on the occurrence of ABB pseudopregnancy (a physiological state in which a non-pregnant bear exhibits progesterone levels similar to gravid bears in the absence of an actual pregnancy). Also, there is little known about postnatal development of cubs or the influence of maternal traits on embryonic implantation and cub growth. Finally, the role of satellite cells (SCs – stem cells able to regenerate muscle fibers) play in maintaining muscle functionality during hibernating remains understudied. Therefore, I aimed to assess these four aforementioned aspects using wild ABBs held temporarily captive at Virginia Tech's Black Bear Research Center (VT-BBRC). The major findings of this dissertation are: 1) I suggest that wild ABBs do not experience pseudopregnancy as a reproductive strategy; 2) interactions between litter size and cub age best described postnatal cub weight dynamics and organ development. Twin cubs were heavier than single and triplet cubs, yet cubs from all litter sizes reached similar weights after mothers began consuming food post hibernation. Single cubs experienced delayed timing in ear, eye, and teeth development compared to other litter sizes; 3) maternal traits such as higher body weight and higher ability to gain weight in the fall are closely associated with earlier timing of embryonic implantation than in leaner females, which gained less weight per day in the fall; and 4) SC ability to generate muscle fibers is increased during ABB hibernation. I propose that maintaining the SCs are an important potential pathway for limiting muscle atrophy during bear hibernation. Understanding pre and postnatal development of ABBs is important for exploring factors related to climate, maternal characteristics, which possibly affect birthing phenology, and fitness of bears experiencing rapid anthropogenic environmental change. Functional aspects of bear muscle conservation are interesting for potentially for elucidating avenues to improve treatments for human metabolic disorders such as muscular dystrophy, sarcopenia, and disuse atrophy.

Abstract (General Audience)

The American black bear (ABB) (*Ursus americanus*) exhibits physiological strategies highly synchronized with the environment. Such strategies enable bears to exploit food resources when available and survive the winter months by hibernating without ingesting food or water. However, there are multiple aspects of ABB hibernation physiology that remain unknown. For instance, there is conflicting evidence on the occurrence of false pregnancies in the ABB. Also, there is little known about postnatal development of cubs or the influence of maternal traits at the beginning of active gestation and cub growth. Finally, the role of satellite cells (SCs – stem cells able to regenerate muscle fibers) play in maintaining muscle functionality during hibernating remains understudied. Therefore, I aimed to assess these four aforementioned aspects using wild ABBs held temporarily captive at Virginia Tech's Black Bear Research Center (VT-BBRC). The major findings of this dissertation are: 1) I suggest that wild ABBs do not experience false pregnancy as a reproductive strategy; 2) interactions between litter size and age best described postnatal cub weight dynamics and organ development. Twin cubs were heavier than single and triplet cubs, yet cubs from all litter sizes reached similar weights after mothers began consuming food post hibernation. Single cubs experienced delayed timing in ear, eye, and teeth development compared to other litter sizes; 3) maternal characteristics such as higher body weight and higher ability to gain weight in the fall are closely associated with earlier timing of embryonic implantation than in leaner females, which gained less weight per day in the fall; and 4) SC ability to generate muscle fibers is increased during ABB hibernation. I propose that maintaining the SCs are an important potential pathway for limiting muscle atrophy during bear hibernation. Understanding pre and postnatal development of ABBs is important for exploring factors related to climate, maternal characteristics, which possibly affect birthing phenology, and fitness of bears experiencing environmental change. Functional aspects of bear muscle conservation are interesting for potentially for elucidating avenues to improve treatments for human metabolic disorders such as muscular dystrophy, sarcopenia, and disuse atrophy.

Attributions

Dr. Janine L. Brown is a research scientist and director of the Endocrine Laboratory of the Center of Species Survival at the Smithsonian Conservation Biology Institute. Dr. Brown has conducted endocrine research to better understand reproductive and stress biology of endangered wildlife, including several felid species, for more than 20 years. Dr. Brown contributed supplies and facilities for hormonal analysis in this work and provided significant input in study design and analytical approaches.

Dr. Sherrie Clark-Deener is an associate professor in the Department of Large Animal Clinical Sciences of the Virginia-Maryland College of Veterinary Medicine at Virginia Tech. Dr. Clark-Deener is a theriogenologist focused on in reproductive health of domestic animals. Dr. Clark-Deener contributed supplies for reproductive hormonal analyzes and assisted bear immobilizations and ultrasound diagnostics.

Dr. Marcella J. Kelly is a professor in the Department of Fish and Wildlife Conservation at Virginia Tech. Dr. Kelly's research program uses population dynamics framed in an ecological context and cutting-edge technologies to promote wildlife conservation. Dr. Kelly has studied felids in Belize for over 12 years and bears for 6 years. Dr. Kelly contributed financial support and provided significant input in study design and analytical approaches.

Colleen Olfenbuttel is the bear/furbearer biologist of the North Carolina Wildlife Resources Commission. Ms. Olfenbuttel has over 10 years of experience as the North Carolina state bear biologist. Ms. Olfenbuttel has curated the data set collected from 1988 until 2009 at Virginia Tech's Black Bear Research Center and provided significant input in study design and analysis of this dissertation.

Dr. Robert Rhoads is an associate professor in the Department of Animal and Poultry Sciences at Virginia Tech. Dr. Rhoads research interests focus on mammalian growth and development with an emphasis on the investigation of cellular and molecular mechanisms governing skeletal muscle size, regeneration, and metabolism. Dr. Rhoads contributed supplies and facilities for skeletal satellite cell analysis in this work and provided significant input in study design and analytical approaches.

Dr. Michael Vaughan is a professor emeritus in the Department of Fish and Wildlife Conservation at Virginia Tech. Dr. Vaughan is the founder of Virginia Tech's Black Bear Research Center. Dr. Vaughan has conducted extensive research on the biology, ecology, and management of bears. Dr. Vaughan contributed historic data (1988-2009) and provided significant input in study design and analytical approaches.

Dr. Lidan Zhao is a research associate working under the direction of Dr. Rhoads at Virginia Tech. Dr. Zhao conducted the bulk of laboratory work related to satellite cells. Dr. Zhao also provided significant input in analytical approaches for Chapter 4.

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1. Introduction

The American black bear (ABB) (*Ursus americanus*) exhibits several physiological strategies highly synchronized with the environment. Such strategies enable bears to exploit a broad range of food resources when available, and survive, via hibernation, without ingesting any food or water during the winter months (Hellgren 1998). Therefore, physiological adaptations of ABBs can be divided in two main phases: active and hibernating.

The use of the terms hibernation and torpor may generate some confusion to biologists due to extensive metabolic and behavioral adaptations exhibited throughout the animal kingdom. In this document, I will refer to torpor as a period where an animal experiences a controlled metabolic depression and reduced physical activity. Torpor is characterized by lower heart, respiratory, and metabolic rates, and body temperature is modified to reach a lower temperature differential between the body and the environment (van Breukelen and Martin 2016). Torpor can last for days or weeks. Torpor during winter is usually known as hibernation, whereas torpor during the warmer months is known as estivation (Lindsay, 2012). In addition, some species such as hummingbirds (*Selasphorus rufus*) and the edible dormouse (G*lis glis*) can experience daily torpor, which is characterized by controlled metabolic depression lasting less than 24 hours (Hiebert 1990, Wilz and Heldmainer 2000).

The active phase in the ABB occurs usually in spring, summer, and fall, in most of their range. At the beginning of the active phase (*e.g.*, early spring), during den emergence, ABBs exhibit an anorexic or hypophagic state known as "walking hibernation", which might extend for two weeks (Nelson et al. 1983). Thereafter, from approximately late April-September, ABBs resume "normal activity" by consuming foods rich in protein allowing them to prepare for the

mating season (approximately June-August). Subsequently, ABBs enter a hyperphagic state, in which foods of high caloric content (high crude fat content) are consumed in the fall (Hellgren et al. 1989). The hyperphagic state allows ABBs to increase corporal fatty deposits in order to "store energy" for overwintering.

Conversely, the hibernating phase [season of torpor with euthermic interbouts longer than 1 day (van Breukelen and Martin 2015)] in ABBs is displayed in habitats that experience winter-like conditions characterized by low environmental temperatures (normally reaching freezing points) and decreased food availability. During the hibernating phase, ABBs reduce their metabolism by 25% of their basal rates, allowing body temperature go from 38°C during the active phase to fluctuate ~33°C during hibernation in 1.6 to 7.3-day cycles (Tøien et al. 2011). This metabolic suppression is accompanied by a drastic decrease in physical activity, anorexia, adipsia, and lack of urination and defecation (Nelson et al. 1983, Hellgren et al. 1990).

Most ursids, and in particular ABBs, are great model species to explore how physiological adaptions allow successful reproduction and offspring survival in changing environments. For instance, ABBs experience delayed implantation, a process manifested soon after mating (in the summer), where fertilized embryos (blastocysts) arrest their development until mid- to late-November when implantation occurs (Lopes et al. 2004). Therefore, gestation in ABBs can be divided in two phases: passive and active. The passive gestation period expands from fertilization until hatching of the blastocyst (~4 months), whereas the active gestation period extends from embryo implantation until parturition (~2 months).

Previous studies conducted on various bear species, including ABBs, have provided evidence suggesting that pseudopregnancy (e.g., false pregnancy) is a natural common

occurrence by showing similar patterns in serum progesterone profiles in females that gave birth and those that did not give birth (Tsubota et al. 1986, Schulz et al. 2003). On the other hand, other studies have suggested that bears that have not mated do not show pseudopregnancy progesterone profiles (Palmer et al. 1988) or might experience fetal loss (Hellgren et al. 1991). In **Chapter 1**, I address whether pseudopregnancy occurs in ABBs using serum P_4 and estradiol profiles, as well as body temperature and hibernation behavioral patterns to determine whether bears that were never detected as pregnant by ultrasonography experience pseudopregnancy or fetal loss.

Newborn ABB cubs have been described as small in relation to the mother's weight compared to other mammals (Case 1978). Some studies, including three Masters theses developed in Virginia at Virginia Tech, have conducted morphological measurements in denning cubs over a single time period, finding no significant differences in morphological measurements between male and female cubs (Godfrey 1996, Ryan 1997, Echols 2000). Body composition and organ weight has been measured in postnatal ABB cubs (Oftedal et al. 1993), but other repeated measures of their growth have not been reported. Thus, very little is known about postnatal growth rates in offspring of this species, possibly due to the denning nature of parturient females (Bridges et al. 2002). Yet, there is a crucial need to understand postnatal cub development to aid in ABB management. For instance, it is important to know general growth rates in cubs to assess the effects of stochastic environmental events and female condition on cub development. Similarly, fostering orphan cubs to surrogate females could be improved with this basic information (Carney and Vaughan 1984, Benson and Chamberlain 2006). **In Chapter 2**, I build on the work of Colleen Olfenbuttel (presented at International Bear Management Association Conference in 2004) by increasing sample sizes and exploring growth dynamics to address

neonatal cub growth rates.

Reproductive parameters of a population may be dictated by physiological characteristics of the species, environmental conditions, and habitat suitability. Species experiencing delayed implantation are considered to be more flexible in their reproductive physiology to exploit resources in a more efficient way to ultimately produce more offspring or offspring with higher survival probabilities (Robbins et al. 2012). For instance, female badgers (*Meles meles*), which also experience delayed implantation, time their implantation date according to their body condition; females with better body condition initiate active gestation earlier in the year (Woodroffe 1995). Moreover, female brown bears (*Ursus arctos*) with higher body fat content experienced parturition earlier in the year and their cubs grew at faster rates than leaner female bears (Robbins et al. 2012). ABBs in the Alleghany Mountains of Virginia tend to increase their litter sizes as they get older and synchronize their reproductive efforts with hard mast production, while younger females tend to give birth later in the year than older bears (Bridges et al. 2011*a*, *b*).

Understanding maternal effects on reproductive performance in ABBs could be useful to determine how environmental stochasticity influences population dynamics and harvest quotas. For instance, a year with hard mast failure could limit fat deposition in females, thus ABBs may produce smaller litter sizes and have decreased cub survival rates (Bridges et al. 2011*a*, *b*). Furthermore, assessing maternal effects on reproductive parameters could be used to assess potential consequences of shifts in birth dates due to rapid anthropogenic changes in the environment, which combined with environmental stochasticity, could potentially increase human-bear conflict or alter black bear population dynamics (positive or negatively impacting human-bear conflict). In **Chapter 3**, I examine the influence of maternal body condition on

birthing phenology by using previous and new information related to maternal age, maternal body mass, maternal serum leptin concentrations, date of parturition, litter size, litter mass, and cub growth rates of bears housed at the BBRC since 1988.

The field of biomimicry (using nature as a model) has recently emerged to find solutions to complicated health issues affecting humans. Bears have also been described as "metabolic magicians" worth examining for sarcopenia (muscle loss) or muscle atrophy (Stenvinkel et al. 2013, Doherty et al. 2014), because bear metabolism seems to deal more efficiently than humans with metabolic byproducts such as urea and ketone bodies. During hibernation, bears experience limited muscle atrophy despite the 3-4 month period of low physical mobility (Lohuis et al. 2007*a*). Therefore, elucidating how bears are able to maintain muscle mass and physiological properties could be key in benefitting humans that may suffer many diseases caused by limited physical activity or limited nutrient intake. During hibernation, ABBs maintain most of their biomechanical skeletal muscle characteristics and only lose about half of the muscle strength that humans would lose under the same conditions (Lohuis et al. 2007*a*, *b*). Skeletal muscle biochemistry is also relatively conserved in ABBs during hibernation, although DNA concentrations have been shown to increase and the ratio of protein DNA decreases during hibernation while RNA concentrations increase significantly post hibernation (Koebel et al. 1991). Those changes indicate that there is potential for an increased capacity for skeletal muscle protein biosynthesis after spring arousal to compensate for the 10-20% muscle fiber atrophy, which is considered minimal atrophy considering the extended period of fasting and inactivity (Fedorov et al. 2009). Nevertheless, to my knowledge, no studies have investigated the role of SCs on muscle conservation during hibernation in ursids. SCs (which received their name based on the peripheral location in relation to skeletal muscle fibers (Mauro 1961)) are important for

postnatal skeletal muscle growth and skeletal muscle regeneration in adults. More recently SCs have been also linked to angiogenic processes (generation of blood vessels) and to proliferation of adipose tissue (Dodson et al. 2010, Rhoads et al. 2013, Murach et al. 2018). Myostatin, a transforming growth factor (a.k.a. TGF-8), is a regulator of SC proliferation and differentiation in domestic animals. Mice and cattle lacking myostatin exhibit an increase in muscle mass due to a proliferation in the number of muscle cells (hyperplasia) (Rhoads et al. 2009*b*). In **Chapter 4**, I focus on satellite cell (SC) activity of bears during the different physiological states and explore associations with serum myostatin concentrations and physical movement measured through accelerometry.

Study Site and Historic Background

These studies took place at Virginia Tech's Black Bear Research Center (VT-BBRC) in Blacksburg, Virginia (17S 549889.34E 4118392N) (Fig. 1.1). The BBRC is one of two facilities in the United States that aids in management of wild black bears while focusing on research relevant to bear biology and human health. The VT-BBRC originated from efforts of Dr. Michael Vaughan and the Virginia Department of Game and Inland Fisheries (VDGIF). In 1988 the BBRC held the first cohort of female bears. Since then, under Dr. Vaughan's direction, the BBRC held 111 bears, mostly females, until 2009 when he retired. During that period, Dr. Vaughan and multiple graduate students contributed substantially to our understanding of bear biology in multiple areas including: reproductive biology, cub aging, bone physiology, hibernation metabolism, immunology, and anesthetic pharmacodynamics (Hellgren et al. 1990, 1991, Bridges et al. 2002, Donahue et al. 2006, Ryan et al. 2009, Chow et al. 2013). Some instrumental graduate students such as Dr. Eric Hellgren and Colleen Olfenbuttel explored new avenues of research and curated the long-term data set containing information related to cubs and

female morphological measurements collected at the VT-BBRC.

In 2012, Dr. Marcella Kelly, from the Department of Fish and Wildlife Conservation, assumed the direction of the VT-BBRC. I have been involved in the operations of the VT-BBRC during the entire period of Dr. Kelly's tenancy. In past years, most bears housed at the BBRC resulted from human-bear conflict situations. That is no longer the case. VDGIF now delivers bears caught for any reason, as deemed necessary by VDGIF, and this may not include bears resulting from human-bear conflict. Today, the facility's purpose is two-fold. First, it meets a sociological need directly linked to addressing human-bear conflict in Virginia. VDGIF responds to bear incidents and takes captured bears and orphaned cubs to BBRC. This provides a mechanism for public outreach and imparts a positive image of the Agency. Second, the BBRC studies bears' unique physiology, which is not only relevant to bear biology, but also to human health.

The VT-BBRC houses wild ABBs, brought by the VDGIF, from July until their release back into the wild in April or May of the following year (Fig. 1.2D). Over this timeframe bears are allowed to acclimate to captive conditions, experience the hyperphagic state, the hibernation phase, parturition, and resumption of the active phase. Bears at VT-BBRC traditionally have been anesthetized every 10 days between October – May to obtain morphological measurements and biological samples such as blood and biopsies (Fig. 1.2). To date the VT-BBRC has housed 130 adult bears, 155 cubs born at the facility, fostered 54 orphan cubs, and compiled morphological measurements, anesthetic information, and obtained over 3,000 serum and plasma samples (Fig. 1.2).

The facility at the VT-BBRC consists of a pole barn with roof to protect animals from

rain, but otherwise allows exposure to natural climate conditions (Fig. 1.3A). Physical location of the barn prevents bear exposure to noise foreign to their natural habitat. The barn contains six circular metal enclosures, 10 feet high and 16 feet in diameter; each one is equiped with a metal culvert denning box (Fig. 1.3). Each bear enclosure has a large wooden scratch pole and straw bedding to provide behavioral enrichment and comfort. Adult bears are housed singly in each enclosure, except in the case of mothers and their cubs. This setting allows a maximum capacity of holding 5 adult bears per season, since one enclosure is left vacant for unexpected damage of cages (Fig. 1.3B).

In this dissertation, I used data collected from bears housed at the BBRC between 1988 and 2016. **Chapter 1** includes information on pregnancy diagnosis, rectal temperature, weight dynamics, and serum hormone analysis from 29 adult females. **Chapter 2** includes information on cub weights and postnatal organ development and maternal food consumption post hibernation from 129 cubs from 58 different adult female bears. **Chapter 3** includes information on weight dynamics and parturition timing from 48 adult females and 112 cubs. **Chapter 4** includes movement, serum hormone analysis and muscle biopsy samples collected from adult bears housed in more recent years (2014-2016). Data from orphan cubs was not included in any of the chapters of this dissertation.

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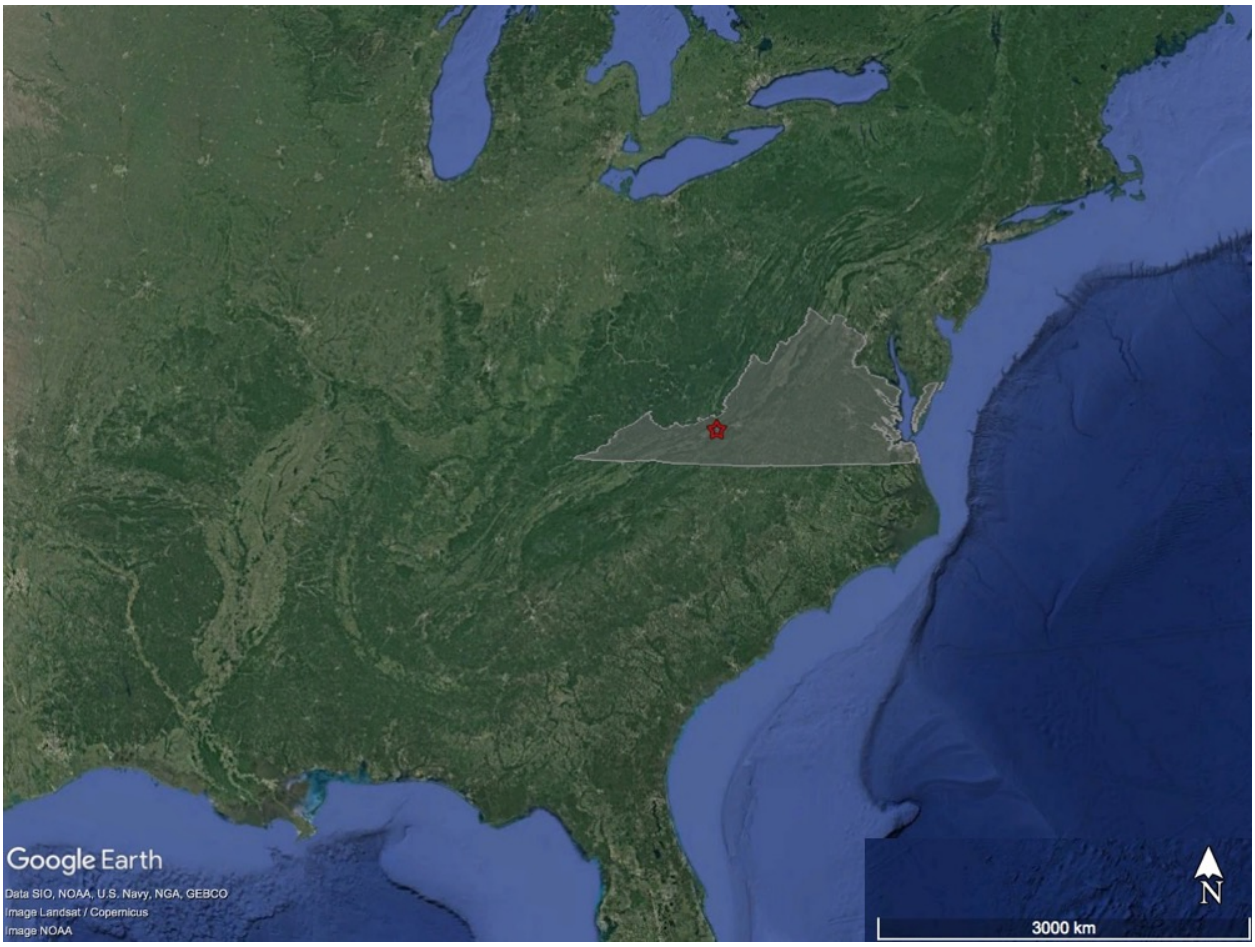

Figure 1.1. Geographic location of Virginia Tech's Black Bear Research Center (VT-BBRC) in Virginia, USA. VT-BBRC: red star and Virginia (grey shaded area). Map layers retrieved from Google Earth, Google Inc. 2018.

Figure 1.2. Activities performed at Virginia Tech's Black Bear Research Center. A. Chemical immobilizations and undergraduate student training. B. Cub developmental monitoring (T. Pangle). C. Blood sample collection from chemically immobilized bears (J. B. Mesa-Cruz). D. Bear release back into the wild by the Virginia Department of Game and Inland Fisheries (VDGIF) (B. Stinson)

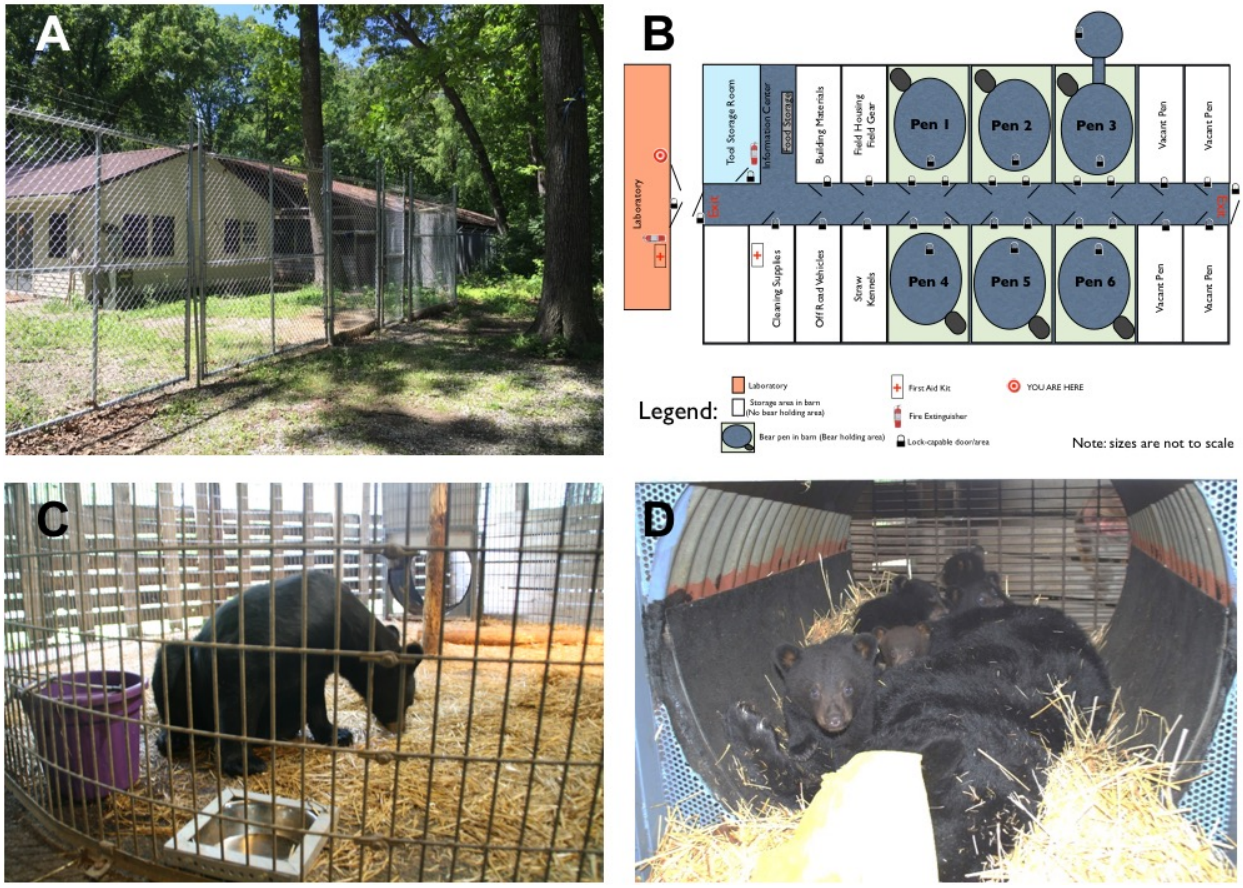

Figure 1.3. Facilities at Virginia Tech's Black Bear Research Center (VT-BBRC). A: Metal enclosure exposed to natural sun light and climate conditions. B. Floor plan of the bear holding area. C. Adult female bear with cubs after female chemical immobilization in denning box.

2. Chapter 1: Physiological and Behavioral Assessment Reveal No Evidence for Pseudopregnancy in American Black Bears (*Ursus americanus*)

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Abstract

The American black bear (*Ursus americanus*) exhibits several reproductive strategies that are highly synchronized with the environment for efficient use of energy resources. It has been reported that black bears experience pseudopregnancy, a physiological state in which a non-pregnant female exhibits progesterone (P_4) levels similar to gravid bears in the absence of an actual pregnancy. Some studies have shown similar patterns in serum P_4 profiles in parturient and non-parturient ABBs. However, pregnant bears experiencing *in utero* fetal death could display similar P_4 levels to those that produced cubs, thereby creating false positives for pseudopregnancy. To further investigate whether black bears exhibit pseudopregnancy, we selected 29 adult females from 89 adult females that were temporarily housed at the Black Bear Research Center at Virginia Tech, USA. We compared P_4, estradiol (E_2), rectal temperatures, body weight, and behavioral changes of ABBs diagnosed by ultrasound and/or giving birth as: pregnant and parturient (P-P), pregnant not parturient (i.e., embryonic death) (P-NP), or not pregnant not parturient (NP-NP). Contrary to previous studies, we found P_4 concentrations of P-P, P-NP, and NP-NP bears to be significantly different. Pregnant (both P-P and P-NP) ABBs showed a 4-fold increase in P_4 after embryonic implantation, while NP-NP ABBs maintained low P_4 levels. P-P and P-NP ABBs also displayed lower E_2 concentrations during pregnancy than before implantation, while E_2 of NP ABBs did not change significantly over time. Rectal temperature of NP-NP ABBs dropped ~2°C during the time period concurrent with active gestation in P-P and P-NP ABBs, 1°C lower on average than P-P and P-NP ABBs. During the hibernating active gestational stage, NP-NP ABBs lost less weight than the other bears, however, there was no difference in proportional weight change among all ABBs. NP-NP ABBs entered hibernation ~15 days later than P-P ABBs, consistent with NP-NP ABBs spending less time hibernating. Our results suggest that wild ABBs do not experience pseudopregnancy as a reproductive strategy.

Introduction

The American black bear (ABB) (*Ursus americanus*) is a good model species to explore how physiological adaptions allow for successful reproduction and offspring survival in changing environments (Sandell 1990). ABBs mate during late spring and early summer, a time when they consume high protein content foods (Hellgren et al. 1989, Spady et al. 2007). Ovulation in this species can be induced by either copulation or presence of a male in the vicinity (e.g., visual/olfactory stimuli) (Boone et al. 1995, 2004). Soon after mating, bears experience delayed implantation, a process by which fertilized embryos (blastocysts) are arrested in their development until late fall (mid- to late-November), after consumption of high fat content foods to increase body fat stores prior to hibernation (Hellgren et al. 1989, Lopes et al. 2004, Spady et al. 2007). Thereafter, embryonic reactivation and implantation occurs to finally carry out *in utero* development of offspring and parturition (birth) occurs during the hibernating phase (typically December through March). Hibernation in adult females is characterized by anorexia, adipsia, and dramatic metabolic suppression (Nelson et al. 1983, Hellgren et al. 1991, Tøien et al. 2011). Gestation in pregnant ABBs can be divided into two stages: passive and active. The passive gestation stage extends from fertilization until hatching of the blastocyst (~100 days; typically late July to late November), whereas the active gestation stage extends from embryo implantation until parturition (~60 days; typically December to January).

Pregnant and non-pregnant ABBs experience different metabolic demands and behavioral responses during the time period that occurs during active gestation stage while hibernating. Pregnant females tend to maintain normal core body temperature (~37.5°C) with little fluctuation during the active gestation stage, possibly to provide an optimal environment for embryonic and fetal development (Tøien et al. 2011). This is also accompanied by higher physical activity

displayed by pregnant females compared to non-pregnant females during the same period (Tøien et al. 2011). Similar to the ABB, pregnant and non-pregnant European brown bears (*Ursus arctos*) also display similar body temperature and activity patterns during the active gestation stage (Friebe et al. 2013, 2014).

It has been reported that black bears undergo pseudopregnancy, defined in carnivores as the maintenance of luteal progesterone (P_4) production, in the absence of pregnancy, for periods and concentrations similar to those of pregnant females, as an obligate stage of the female reproductive cycle after ovulation (Palmer et al. 1988, Hellgren et al. 1991, Sato et al. 2001). Females experiencing pseudopregnancy can also express behaviors and physiological changes similar to those of pregnant females, such as nesting in the giant panda (*Ailuropoda melanoleuca*), lactation in the dwarf mongoose (*Helogale parvula*), or both nesting and milk production in the domestic dog (*Canis familiaris*) (Creel et al. 1991, Steinman et al. 2006, Tsutsui et al. 2007). It is thought that the *corpus luteum* of ursids is pre-programmed to be active for the entire gestational length whether embryos are present or absent in the uterus, making this process advantageous because it ensures that the uterus is ready for implantation at the most appropriate time (Spady et al. 2007). Although there are no reports of P_4 sources during pregnancy in black bears, it is likely that the main source of P_4 is the *corpus luteum* (Tsubota et al. 1998), as formerly reported in the domestic dog (Concannon et al. 1989).

Previous studies conducted on various bear species during the mating season in captivity, including black bears, have suggested that pseudopregnancy is a natural, common occurrence (Tsubota et al. 1986, Sato et al. 2001, Stoops et al. 2012). To date, however, there is no evidence that free-ranging bears experience pseudopregnancy (Palmer et al. 1988, Harlow et al. 1990, Tsubota et al. 1998). Moreover, most studies providing evidence of pseudopregnancy in ABBs

did not include extensive monitoring of fetal development during the active gestation stage, which could have resulted in false negative pregnancy observations. For instance, Sato et al. (2001) and Hellgren et al. (1991) performed pregnancy ultrasounds once after the second half of the active gestation stage. Thus, pregnant females that experience *in utero* fetal death during the first half of the active gestation stage could display similar P_4 profiles to those that produced live offspring, and consequently could be misinterpreted as pseudopregnancy. Therefore, we aimed to assess whether ABBs that were available during the mating season in the wild exhibit pseudopregnancy by comparing serum P_4 and estradiol (E_2) concentrations, rectal temperature, timing of hibernation onset, and changes in body weight during the entire active gestational stage of females as diagnosed by transabdominal ultrasound.

Methods

In the late summer or early fall of each year, after the breeding season, free-ranging adult female ABBs without cubs were captured from the wild by the Virginia Department of Game and Inland Fisheries (VDGIF) and temporarily housed at Virginia Tech's Black Bear Research Center (BBRC) between 1989 and 2016, as previously described in Hellgren et al. (1990). During this period, we individually housed a total of 89 adult females, of which 62 bears were parturient (69.6%) and 27 (31.4%) did not give birth or no evidence of parturition was observed (Table 1). The VDGIF released all bears back into the wild the following spring. All procedures were previously approved by the Institutional Animal Care and Use Committee at Virginia Tech under protocols 98-069, 09-073, 12-112, and 15-162.

Feeding Protocol

We used a feeding regimen that closely mimics energy resource consumption in the wild (Nelson et al. 1983, Hellgren et al. 1990). We provided bears with dry dog food at ~40 kcal/kg/d

between arrival and November 1st, we then increased the food from November 2nd until December 7th, to ~80 kcal/kg/d. Thereafter, we reduced the food available by 1-fold per week until the first week of January, when food was completely removed if the bear had not stopped food consumption voluntarily. Food was slowly reintroduced to bears arousing from hibernation at the end of March or beginning of April, or earlier if bears showed signs of hibernation arousal such as: defecating, increased physical activity, and water consumption. We offered water to bears *at libitum* during the extent of the study.

Sample Size

For this study, we included a subsample of the total number of females to conduct physiological and behavioral analyzes (as described in the sections below). We used the following criteria to create the subsamples: a subsample of 10 females that were diagnosed as pregnant and experienced parturition (P-P) between 2003 and 2005, and median age 5 years (Min = 3; Max = 20) , a subsample of 10 females diagnosed as not pregnant and not parturient (NP-NP) between 1989 and 2014, and median age 7 years (Min = 3; Max = 20), and all 9 females that were pregnant but were not parturient (P-NP), due to *in utero* fetal death, between 1989 and 2002, and median age 6 years (Min = 2; Max = 19) (Table 1).

Obtaining Physiological Information

We chemically restrained bears every 10 days with an intramuscular administration of a ketamine (4 - 8 mg/kg), xylazine (0.8 - 2 mg/kg), and occasionally a Telazol® (1.5 mg/kg) mixture to obtain blood samples, rectal temperature (°C), and body measurements, including weight (kg) from October until the subsequent spring (e.g., May). We measured ambient temperature (°C) (Lascar Electronics EL-USB-2) simultaneously with bear rectal temperatures in

the sampling season of 2015-2016 (4 bears) to determine whether rectal temperature was influenced by ambient temperature. We also performed transabdominal pregnancy diagnosis via B-mode ultrasound (Aloka ecocamera SSD-500V and a 3.5MHz prove Hitachi-Aloka Medical America Inc.) from November to February. Bear ages were assessed through dental cemetum layer analysis (Willey 1974) by Matson's Laboratory LLC (MN, USA). We measured serum P_4 and E_2 using commercially available radioimmunoassay kits (ImmuChem™, MP Biomedicals, LLC. Orangeburg, NY). Immunoassay validations for ABB serum included a linearity test (P_4: $F_{1, 9} = 0.0217$, $P = 0.8999$; E_2: $F_{1, 1} = 0.537$, $P = 0.5856$) and accuracy recovery assessment (P_4: mean = 99.8%, SE = 5; E_2: mean = 99.3%, SE = 21.9), as described by Mesa et al. (2014). The inter-assay variation for P_4 was 12.91% and 7.28% for E_2, and intra-assay variation was < 10% for both hormones.

We determined gestational stages for non-pregnant bears based on parturition dates of pregnant bears housed at the BBRC (n = 62). We used a 95% confidence interval (CI) to determine the range of days when bears underwent parturition. The peri-implantation period (e.g., surrounding time of embryo attachment to endometrium corresponding to the transition from passive to active gestation stages) was established by backtracking 60 days from parturition, as this is the suggested active gestation length in ABBs (Hellgren et al. 1991). The active gestation stage was stablished as the latest date of implantation and the latest date of parturition. The pre-implantation period (e.g., passive gestation stage) was established as the initial date of sampling (~October 1st) until the earliest date of implantation. Postpartum was determined as the period after the latest parturition date until early- to- mid-March.

Behavioral Observations

We conducted visual inspections consisting of sessions that lasted between 15 and 30 minutes twice a day (morning and afternoon), to document physical activity, food and water consumption, denning onset for hibernation, and evidence of parturition. Cub cries and visual detection of cubs determined time of parturition.

Statistical Analyzes

We employed linear mixed models (LMM) using the restricted maximum likelihood (REML) approach to detect differences in hormone concentration and rectal temperatures between the fixed effects pregnancy status (parturient, pregnancy loss, and non-pregnant) and gestation stage (pre-implantation, peri-implantation, active gestation, and postpartum). Models included bear unique identification as a random effect to account for repeated measures from the same individual. We used analysis of variance (ANOVA) to compare timing of hibernation onset and weight loss during the active gestation stage while hibernating across the three different pregnancy statuses (pregnant and parturient: P-P; pregnant not parturient: P-NP; and not pregnant not parturient: NP-NP). We determined correlation coefficients of rectal and ambient temperatures, in a subsample of 4 bears to determine whether ambient temperatures influenced our rectal temperature measures. *Post hoc* Tukey HSD tests were employed when appropriate ($\alpha = 0.05$). Results are presented as means ± standard error of the mean (SE), unless otherwise noted. All analyses were implemented in the statistical software JMP® Pro (version 13.0.0, SAS Institute Inc.).

Results

Serum Progesterone(P_4) and Estradiol (E_2) for Subsample of 29 Bears

We analyzed P_4 and E_2 in 370 serum samples from the 29 adult bears subsampled for this part of the study. P_4 concentrations of P-P, P-NP, and NP-NP bears were significantly different ($F_{2, 32.4}$ = 5.184, P = 0.01). This difference was mainly driven by a 4-fold increase in P_4 of pregnant bears (P-P and P-NP) from ~4 ng/mL to ~15 ng/mL during the active gestation stage, whereas NP-NP bears maintained relatively low P_4 levels that did not change significantly over time (Fig. 2.2A, Fig. 2.1D, and Fig. 2.1F). Overall, pregnant bears exhibited a significant gradual increase in P_4 ($F_{3, 345.7}$ = 23.524, P < 0.001) from the pre-implantation stage (P-P: 5.11 ± 1.66 ng/mL; P-NP: 3.48 ± 1.37 ng/mL) through active gestation (P-P: 15.02 ± 1.18 ng/mL; P-NP: 9.55 ± 1.16 ng/mL) followed by a drastic decrease in P_4 (P-P: 0.21 ± 2.10 ng/mL; P-NP: 0.33 ± 1.72 ng/mL) after parturition or after fetal death was diagnosed (Fig. 2.2A, Fig. 2.1D, and Fig. 2.1E). Even though, NP-NP bears displayed a 2-fold increase in P_4 concentrations during the peri-implantation and active pregnancy stages from 1.75 ± 1.62 ng/mL to 3.39 ± 1.16ng/mL, the changes were relatively minor and not significantly different (*post hoc* Tukey HSD: P > 0.05) (Fig. 2.2A and Fig. 2.1F).

P-P bears exhibited the highest concentrations of E_2, although we did not find any significant differences across bears with different pregnancy status ($F_{2, 27}$ = 1.782, P = 0.1875) (Fig. 2.2B). Furthermore, E_2 significantly decreased from ~30 pg/mL before birth to ~18 pg/mL postpartum or after fetal death was diagnosed ($F_{3, 334.4}$ = 5.811, P < 0.001) (Fig. 2B and Fig. 1D). NP-NP bears displayed similar E_2 concentrations through time (26 ± 5.15 pg/mL), even after the time when P-P bears experienced parturition (Fig. 2.2B and Fig. 2.1F).

Rectal Temperature for Subsample of 29 Bears

Ambient temperature and rectal temperature exhibited a significant weak positive correlation (r = 0.386, n = 66, $P = 0.0014$). Overall bears displayed a decreasing trend in rectal temperature of 2 ± 0.13°C from pre-implantation to postpartum, however temperature decreased differently across bears of different pregnancy status ($F_{2, 23.3} = 5.506$, $P = 0.011$) and at different gestation stages ($F_{2, 400.2} = 134.743$, $P < 0.001$) (Fig. 1.3A). All bears displayed similar rectal temperatures prior to implantation (37.1 ± 0.12°C), yet during the active gestational stage, rectal temperature was lower for NP-NP by 1.6°C and 0.8°C for P-P and P-NP females, respectively (Fig. 2.3A, Fig. 2.3B, Fig. 2.3C, Fig. 2.3D, and Table 2.1). Additionally, during the active gestation stage, NP-NP bears maintained this significantly lower rectal temperature (~1°C) than P-P bears (*post hoc* Tukey HSD: $P < 0.05$) (Fig. 2.3A). In the postpartum period, all bears exhibited lower rectal temperatures than during other time periods, but in contrast to the active gestational stage, pregnant bears decreased their temperature more (e.g., by 0.9°C for P-P and by 0.4°C for P-NP) than NP-NP females) (Fig. 2.3A, Fig. 2.3B, Fig. 2.3C, Fig. 2.3D, and Table 2.1).

Body Weight Changes

Bears exhibited significant differences in average body weight prior to the implantation period ($F_{2, 119} = 8.885$, $P < 0.001$). This difference was mainly driven by the lower average body weight of P-NP bears (70.4 ± 4.4 kg) compared with both NP-NP (90.3 ± 4.3 kg) and P-P bears (96.1 ± 4.7 kg) (Table 2.1).

After implantation occurred and bears entered hibernation, total weight loss was significantly different across bears of different pregnancy status during the hibernating active

gestation stage ($F_{2, 28}$ = 5.936, P = 0.008) (Fig. 2.4B). NP-NP and P-NP bears lost about 7 kg of body weight, while P-P bears lost twice as much weight during this stage (Fig. 2.4B and Table 2.1). Nevertheless, we did not observe a significant difference in proportional body weight loss (e.g., proportion of weight loss with respect to body weight at hibernation onset) across females during the hibernating active gestational stage ($F_{2, 28}$ = 1.373, P = 0.2726) (Fig. 2.4A and Table 2.1).

Onset of Hibernation

Bears displayed significant behavioral differences in timing of hibernation onset based on pregnancy status ($F_{2, 28}$ = 4.21, P = 0.027) (Table 2.1). In fact, P-P bears entered hibernation, by voluntarily stopping food consumption, before food was removed (median: Dec 22nd), and they arrested physical activity 21.7 ± 3.4 days after the estimated time of implantation and 14.8 ± 5.1 days earlier than NP-NP bears (Table 2.1). P-P bears spent, on average, 39.5 ± 3.5 days of the active gestation stage hibernating, whereas, NP-NP females spent only 24.6 ± 3.7 days hibernating during the same period (Fig. 2.4C and Table 2.1). Additionally, NP-NP bears entered hibernation only when our feeding regimen ended (e.g., all food was completely removed). Hibernation onset for P-NP bears was not significantly different from P-P or NP-NP bears (*post hoc* Tukey HSD: $P > 0.05$), despite voluntarily stopping food consumption after food available started to be reduced, and arresting physical activity, but showing a delay of 7 days compared to the onset of hibernation for P-P bears (Fig. 2.4C).

Discussion

Pseudopregnancy in wild carnivores has been well documented in cooperative breeding species. For instance, in the Ethiopian wolf (*Canis simensis*) and the dwarf mongoose,

subordinate females undergo spontaneous ovulations and subsequent pseudopregnancies, ultimately leading to a higher number of females lactating in the social group to increase the chance of offspring survival after birth (Creel et al. 1991, Kesteren et al. 2013). Pseudopregnancy has been reported in long-term captive bears, but not in ABBs (Stoops et al. 2012, Shimozuru et al. 2013). It is possible that those bears are receiving cues that allow them to ovulate spontaneously and therefore experience pseudopregnancy. For instance, female captive polar bears that are fed year around, appeared to have ovulated spontaneously and showed progestagen profiles similar to those of pregnant females in the absence of an actual pregnancy (Stoops et al. 2012) . Captive Japanese black bears (*Ursus thibetanus japonicus*) display pseudopregnancy after a failed mating or sensory stimulus due to the presence of a male in the breeding season (Shimozuru et al. 2013). Interestingly, those pseudopregnant Japanese black bears also maintained higher body temperatures similar to pregnant females in the active gestational stage during hibernation (Shimozuru et al. 2013).

Our study employed physiological metrics and behavioral aspects of hibernation to investigate whether female ABBs, that were available for mating in the wild, experienced pseudopregnancy. Contrary to previous studies, our data provide little support for the exhibition of pseudopregnancy in free-ranging ABBs. While we did find "pseudopregnant" serum P_4 profiles in some bears that did not give birth, they were determined to have actually been pregnant during the active gestational stage, but later experienced *in utero* fetal loss, as has been previously been suggested (Hellgren et al. 1991).

To our knowledge this is the most extensive active pregnancy monitoring study for hibernating bears. Ultrasound techniques revealed pregnancy losses during the active gestational stage in 12.7% (n = 9) of the 71 pregnant females available in this study. Unfortunately, it is

difficult to compare this rate of pregnancy loss to other species because there is little to no information regarding pregnancy loss rates in other ursids or in any other wild carnivore. This lack of information is primarily due to logistical constraints in handling individuals for sample collection or ultrasound assessments during the active gestational stages. Nevertheless, free-ranging polar bears (*Ursus maritimus*) can experience 33% offspring loss (e.g., either by pregnancy loss during active gestation or after birth during the denning season) (Derocher et al. 1992). Rates of pregnancy loss differ greatly across other taxa; for instance, domestic cattle (*Boss taurus*) have pregnancy losses around 5% of the time, whereas, water buffalo (*Bubalus bubalis*) pregnancy losses have been reported much higher, totaling about 30% (Baxter and Ward 1997, Ahmad et al. 2017). Causes of pregnancy loss in eutherians are many fold and could be related to social structure, infectious agents, toxicants, or nutritional unbalance (Guinet et al. 1998, Kustritz 2005, Panter and Stegelmeier 2011, Henry et al. 2013, Hou et al. 2016).

The median parturition date in our female bears (Jan 28th) was similar to the parturition dates in a free-ranging population in the Allegheny mountains, Virginia (Jan 22nd) (Bridges et al. 2011*b*). In our study, we determined pregnancy length as 60 days, as previously suggested based on serum P_4 raise (Hellgren et al. 1990). It is important to note that our pregnancy diagnosis via ultrasound was confirmatory only after 41 days before parturition (e.g., 19 days or later into pregnancy), thereby creating some uncertainty of pregnancy viability during the first third of the active gestational phase. However, recent evidence indicates that the brown bear and giant panda experience implantation 5 and 40 days after the rise in progesterone, respectively (Friebe et al. 2014, Kersey et al. 2016). Thus, it is possible that implantation in the ABB also occurs a few days after the raise in P_4, which would decrease our window of uncertainty for pregnancy diagnosis during the first third of the active gestational phase.

We observed ranges in body temperatures, before and during hibernation, similar to previous reports (Tøien et al. 2011, 2015, Shimozuru et al. 2013). But, we observed different dynamics in body temperature in P-P bears during the active gestational stage. P-P and P-NP bears exhibited a 1°C decrease in their average rectal temperature during the transition from physical activity to hibernation. It is possible that the use of rectal temperatures may not accurately reflect core body temperature due to anatomical heterothermy in distal parts of the body caused by low environmental temperatures during winter, and second, the anesthetic drugs used to induce chemical immobilization to collect physiological data may have slightly altered thermoregulatory abilities in our bears. Nevertheless, we observed a weak correlation coefficient between ambient and rectal temperatures and rectal temperatures were sampled consistently among all bears with different pregnancy statuses. Our results show that P-P and P-NP females displayed higher rectal temperatures than NP-NP females during the active gestational stage while hibernating; a result consistent with other studies showing that pregnant bears maintain higher core body temperature than non-pregnant females, likely to provide an optimal uterine environment for offspring development (Tøien et al. 2011, Shimozuru et al. 2013).

P-P bears lost more body weight than NP-NP bears during the active gestational stage (although not more proportionally). It is likely that P-P bears need to allocate more energy to thermoregulation, during active gestational stage in hibernation, with the tradeoff of losing more body weight, possibly associated with use of body fat. Another study also found that pregnant bears lost more adipose body stores than non-pregnant bears, possibly due to maintaining higher body temperature during the active gestational period (Harlow et al. 2002).

We detected differences in the onset of hibernation behaviors across pregnant and non-pregnant bears. P-P bears entered hibernation significantly earlier than NP-NP bears, as

previously observed in the wild in hibernating ursids, including the ABB (Schwartz et al. 1987, Friebe et al. 2013). It is also known that cues for hibernation in ursids are multifactorial and may include: climate, food availability, and body condition, among others (Schwartz et al. 1987, Beckmann and Berger 2003, Friebe et al. 2014, Gray et al. 2016). Interestingly, pregnant bears entered hibernation despite having food available for consumption, whereas non-pregnant bears consumed food until we removed the food supply. Moreover, our data show a close relationship between the timing of hibernation and our diagnosis of positive pregnancies via ultrasounds in pregnant bears. It is possible that physiological signals by cytokines or acute phase inflammation proteins associated with implantation could be an additional cue to pregnant bears to seek a den and enter hibernation. Unfortunately, we know little about implantation mechanisms in ursids, so future exploration of the involvement of cytokines and acute phase inflammation proteins in maternal recognition and implantation could allow us to determine direct links between implantation and onset of hibernation in pregnant ABBs (Schäfer-Somi 2003, Kersey et al. 2016).

Interestingly, P-NP experienced physiological patterns in the mid-range between P-P or NP-NP bears, as P-NP bears did not differ significantly from these bears in rectal temperature and body weight loss during the different gestational stages. It is possible that P-NP bears, which were significantly lighter in body weight, did not have sufficient energy stores (e.g., adipose stores) to be able to maintain normal body temperatures or start mammary gland activity for milk production prior to parturition (Olfenbuttle and Vaughan pers. observation). Therefore, they may have lost less weight than P-P females by suppressing their metabolism and maintaining intermediate body temperatures, which possibly resulted in an inadequate environment for offspring development *in utero*. Thus, we speculate that poor body condition may have caused

pregnancy loss in our study due to the significant lower body weight of P-NP females at the onset of hibernation. Evidence of constraints in body condition associated with pregnancy loss has been shown in other species, including those with delayed implantation, such as the African fur seal (*Arctocephalus pusillus*) (Guinet et al. 1998). Future studies could determine the association of body condition with pregnancy loss in the ABB (Harlow et al. 2002, Spady et al. 2009)

Taking our results and other reports of pseudopregnancy in hibernating bears into consideration, the occurrence of pseudopregnancy in free-ranging ABBs would require non-pregnant females that have ovulated to enter hibernation earlier in the year, maintain higher body temperatures, and use more energy from fat stores. Hence, there is no benefit for wild bears to exhibit pseudopregnancy during a period lacking energy resources to ultimately not attain the advantages of reproductive success. Pseudopregnancy in free-ranging ABBs is potentially less likely than in other non-hibernating bears because ABBs have been recognized as metabolic marvels with highly adapted specialized metabolic strategies for using energy efficiently to increase reproductive success (Nelson et al. 1983, Stenvinkel et al. 2013). Therefore, contrary to previous notions, we provide evidence that pseudopregnancy might be rare or does not occur in free-ranging ABB females.

Non-hibernating bears might have slight physiological modifications in their reactivation of corpus lutea around implantation time, but overall there is a major lack of understanding of reproductive physiology in these species. Giant pandas, a species better understood than ABBs, experience pseudopregnancy as an obligate stage in females with active ovarian cycles (Kersey et al. 2010, Willis et al. 2011), but there are great differences in their gestational length and the lack of hibernation may distance these bears' reproductive physiology from other ursids (Kersey

et al. 2016). Moreover, no evidence of pseudopregnancy or seasonal ovarian activity has been observed in the sun bear (*Ursus malayanus*) (Schwarzenberger et al. 2004). Therefore, it is possible that we observe a particular reproductive adaptation in some ursids but not in others, hence we should be cautious about extrapolating physiological and behavioral reproductive strategies across ursids.

While this study increases our understanding of pregnancy physiology of ABBs, further research exploring direct causes of embryonic and fetal death, mechanism of *corpora lutea* reactivation in the fall, implantation mediators, and mechanisms associated with metabolic regulation in hibernating pregnant females are warranted in the ABB.

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Tables and Figures

Table 2.1. Summary of characteristics and timelines of female American black bears available and included in this study. * NP-NP and P-P bears included in the study were randomly selected from the total number of available females, while NP included all the bears in the study that experienced *in utero* fetal death.

Characteristics of Female American Black Bears	Pregnancy Status			Total
	Pregnant-Parturient (P-P)	Pregnant-Not Parturient (P-NP)	Not Pregnant-Not Parturient (NP-NP)	
***Total available number of females:* (N)**	62	9	18	89
Age: min, median, max (years)	2, 8, 20	2, 6, 19	2, 7, 20	
***Number of females included in study*:* (n)**	10	9	10	29
Age: min, median, max (years)	3, 5, 20	2, 6, 19	3, 7, 20	
Gestation Stage Timeline				
Estimated pre-implantation: (dates)	[Oct 1st, Nov 21st]	-	-	
Estimated peri-implantation: median, [95%CI] (dates)	Nov 30th, [Nov 22nd, Dec 4th]	-	-	
Parturition: median, [95%CI] (dates)	Jan 28th, [Jan 23, Feb 8th]	-	-	
Estimated postpartum: (dates)	After Feb 8th	-	-	
Hibernation Timeline				
Onset: median, [95%CI] (dates)	Dec 22nd, [Dec 12th, Jan 2nd]	Dec 29th, [Dec 22nd, Jan 4th]	Jan 2nd, [Dec 22nd, Jan 13th]	
Days hibernating during active gestation: mean, [95%CI] (days)	39.5, [32.2, 46.7]	32.1, [24.0, 40.2]	24.7, [17.0, 32.3]	
Rectal Temperatures				
Pre-implantation: mean, [95%CI] (°C)	36.65, [36.47, 36.97]	37.43, [37.15, 37.59]	37.07, [36.72, 37.41]	
Active gestation: mean, [95%CI] (°C)	35.88, [35.64, 36.14]	36.51, [36.26, 36.77]	35.46, [35.09, 35.82]	
Postpartum: mean, [95%CI] (°C)	34.67, [34.26, 35.02]	35.63, [35.23, 35.99]	35.10, [34.74, 35.52]	
Body Weights				
Pre-implantation weight: mean, [95%CI] (kg)	96.1, [86.7, 105.5]	70.4, [61.6, 79.2]	90.3, [81.7, 98.9]	
Weight loss during active gestation: mean, [95%CI] (kg)	13.3, [10.2, 16.4]	6.6, [3.2, 10.1]	6.9, [3.7, 10.3]	
Proportion weight loss hibernating during active gestation: mean, [95%CI] (%)	11.9, [8.6, 15.3]	9.2, [5.4, 12.9]	8.2, [4.6, 11.69]	

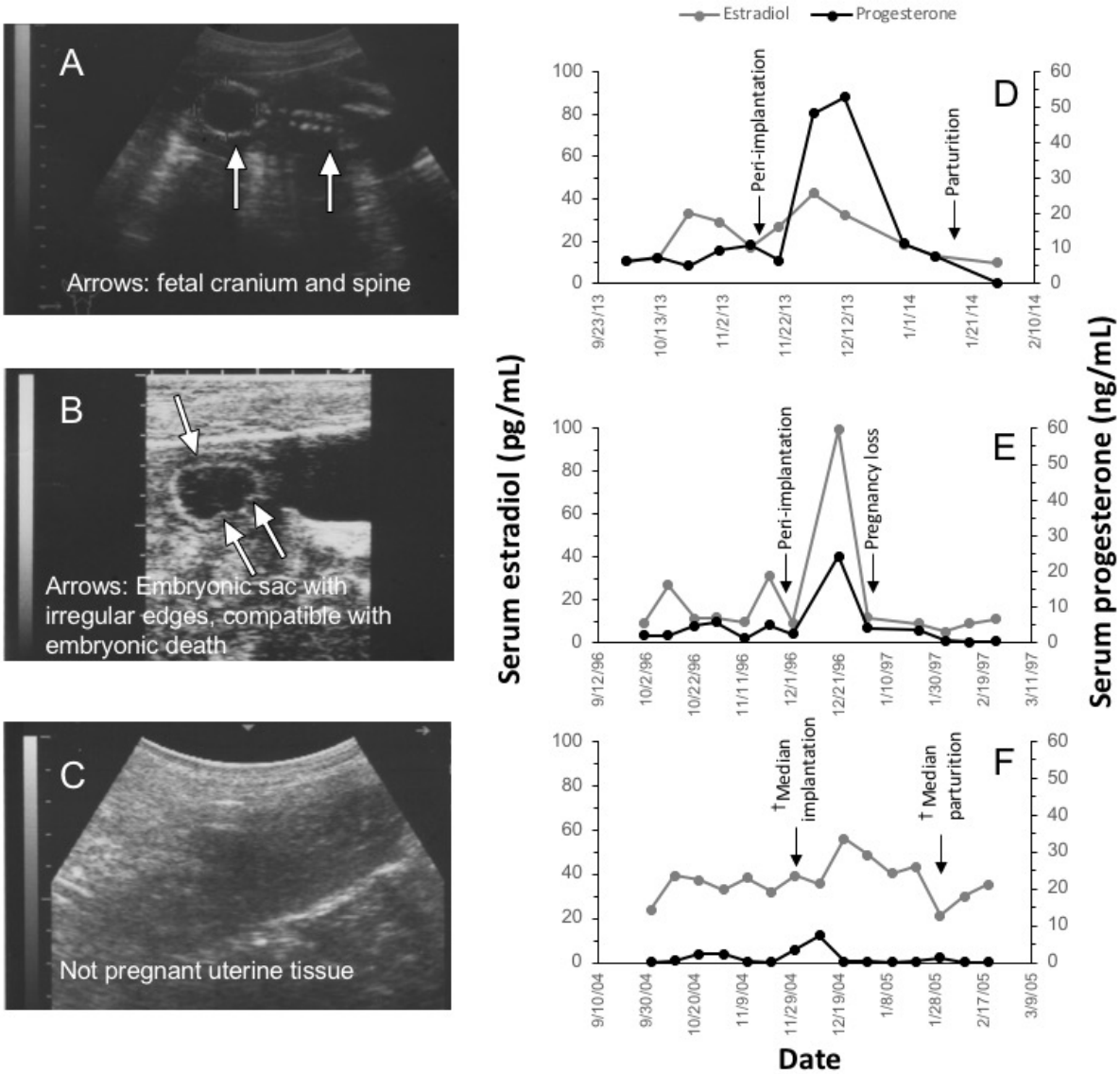

Figure 2.1. Reproductive ultrasound and hormone profiles of female American black bears in three pregnancy statuses. A. Transabdominal ultrasound of a positive pregnancy; arrows denote fetal cranium and spine in a close to term fetus; B. Transabdominal ultrasound of a positive pregnancy with irregular edges on embryonic sac compatible with in utero embryonic death; arrows denote irregular edges of embryonic sac; C. Transabdominal ultrasound of a negative pregnancy diagnosis; D. Typical serum estradiol and progesterone profiles of a pregnant and parturient bear (P-P); E. Typical serum estradiol and progesterone profiles of a pregnant bear that did not experience parturition due to fetal death *in utero* (P-NP); F. Typical serum estradiol and progesterone profiles of a bear diagnosed as not pregnant and did not experience parturition (NP-NP). † = dates of gestation stages for NP-NP were estimated based on dates from P-P bears.

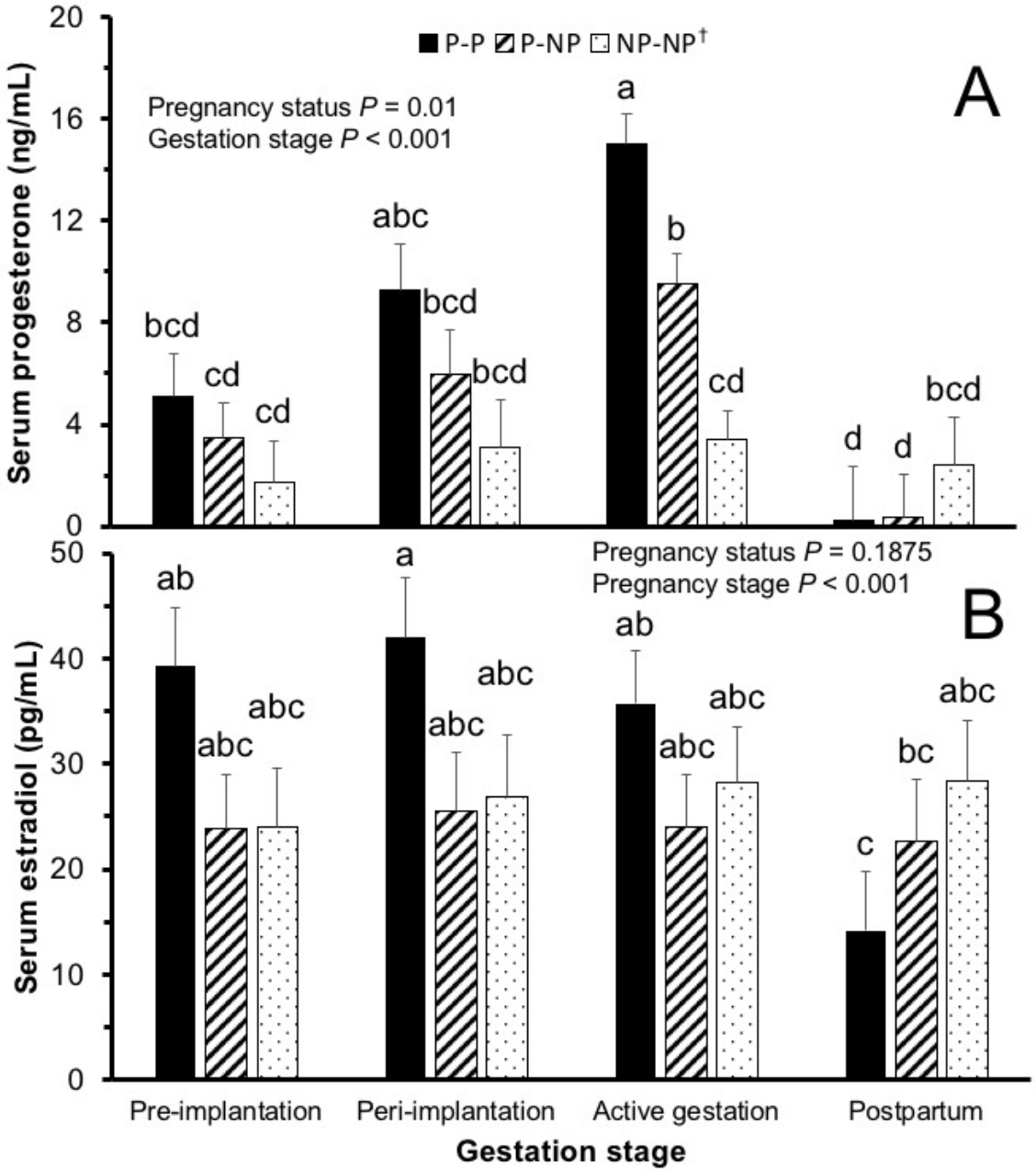

Figure 2.2. Average serum hormone concentrations of female American black bears diagnosed as pregnant and parturient (P-P), pregnant and not parturient due to fetal death *in utero* (P-NP), and not pregnant and did not experienced parturition (NP-NP) over different gestation stages. A. Average serum progesterone; B. Average serum estradiol. Error bars are 1 standard error from the mean. Bars denoted with different letters are significantly different from each other, Tukey's Honest Significant Difference (HSD) test ($\alpha = 0.05$). Pregnancy status = NP-NP, P-P, or P-NP. Gestation stages = pre-implantation, peri-implantation, active gestation, and postpartum. † = dates of pregnancy stages for NP-NP were estimated based on dates from P-P bears.

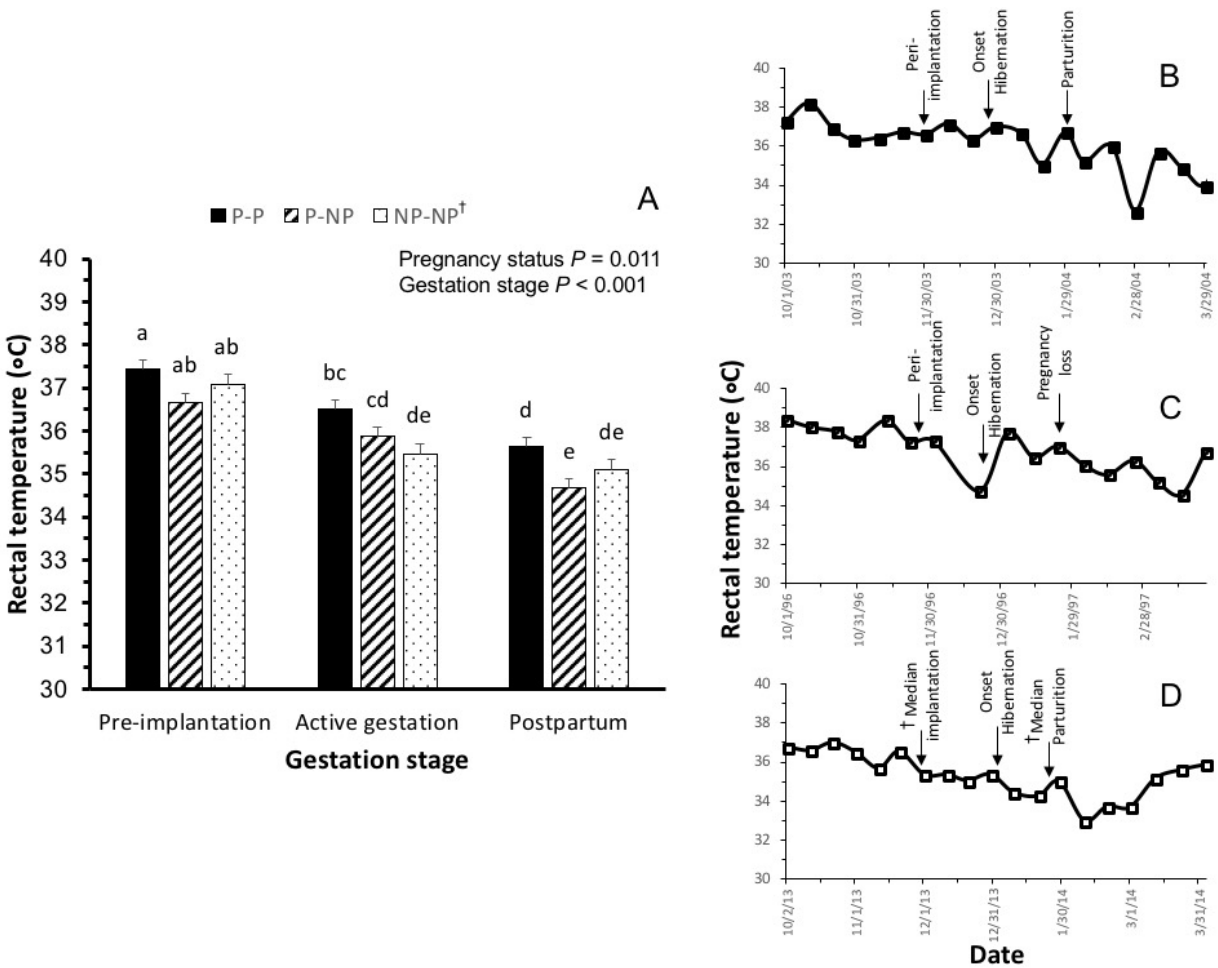

Figure 2.3. Rectal temperatures of female American black bears diagnosed as pregnant and parturient (P-P), pregnant and not parturient due to fetal death *in utero* (P-NP), and not pregnant and did not experienced parturition (NP-NP). A. Average rectal temperature over different gestation stages. Error bars are 1 standard error from the mean. Bars denoted with different letters are significantly different, Tukey's Honest Significant Difference (HSD) test ($\alpha = 0.05$). Pregnancy status = P-P, P-NP, or NP-NP. Gestation stages = pre-implantation, peri-implantation, active gestation, and postpartum. B. Typical rectal temperature profile of a P-P bear. C. Typical rectal temperature profile of a P-NP bear. D. Typical rectal temperature profile of a bear NP-NP bear (NP-NP). † = dates of gestation stages for NP-NP were estimated based on dates from P-P bears.

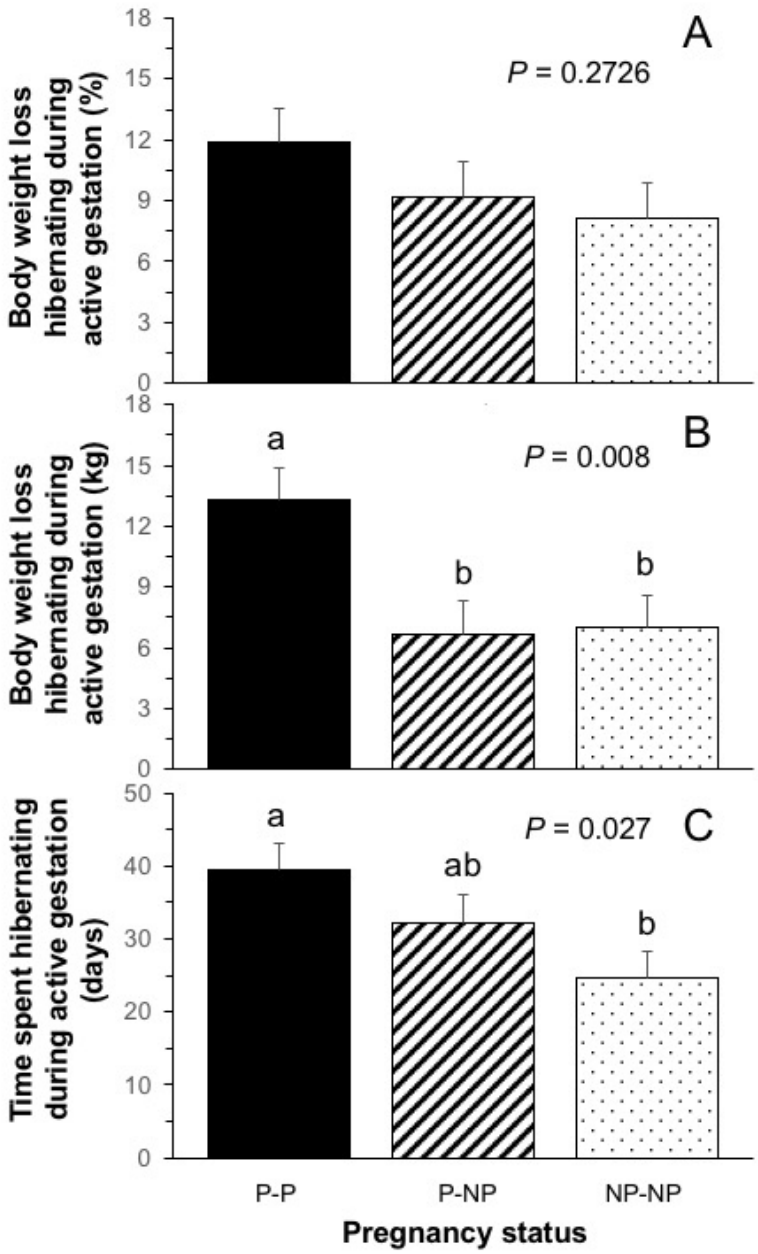

Figure 2.4. Behavioral and body weight responses to hibernation during the active gestation stage of female American black bears diagnosed as pregnant and parturient (P-P), pregnant and not parturient due to fetal death *in utero* (P-NP), and not pregnant and did not experienced parturition (NP-NP). A. Average proportional body weight loss from the onset of hibernation until parturition; B. Average total body weight loss while hibernating during the active gestation stage; C. Average days spent hibernating during the active gestation stage. Error bars are 1 standard error from the mean. Bars denoted with different letters are significantly different, Tukey's Honest Significant Difference (HSD) test ($\alpha = 0.05$). † = dates of gestation stages of NP-NP were estimated based on dates from P-P bears.

3. Chapter 2: Postnatal Development of American Black Bears: Litter Size and Bimodal Weight Gain Phases Influence Growth and Development of Dental and Sensory Organs.

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Abstract

Little is known about postnatal development in American black bear (ABB) cubs (*Ursus americanus*). Understanding early postnatal development of ABB is important for exploring factors related to climate, maternal body condition, and litter characteristics, which possibly affect birthing phenology and fitness of bears experiencing rapid environmental change. At Virginia Tech's Black Bear Research Center (VT-BBRC), we obtained cub measurements every 10 days from the time of birth in January-February until release of bear families back to the wild in April-May. Using linear mixed modeling, we assessed the effects of cub sex, litter size, and age on body weight (BW), daily weight gain (DWG), proportion daily weight gain (PDWG), opening of ears and eyes, and deciduous teeth eruption on 129 cubs born from 58 litters at the VT-BBRC. We found that interactions between litter size and age best described BW, DWG, PDWG, and postnatal organ development of cubs. Overall, recently born cubs (0-9 days old) weigh 0.443 ± 0.079 kg and increased in weight over 9-fold (4.084 ± 0.102 kg) in almost 14 weeks. Twin cubs were heavier than single and triplet cubs, yet cubs from all litter sizes reached similar weights after mothers began consuming food post hibernation. Cubs exhibited two PDWG patterns, where recently born cubs showed a faster growth phase (PDWG > 3.5%) than after 6 weeks of age when growth was slower (PDWG < 3.5%). Triplets showed lower DWG and PDWG during the early, faster growth phase but were able to surpass DWG of twin cubs during the later slower growth phase. We also found that cubs opened their ears and eyes concurrently at 44.07 ± 1.84 and 44.63 ± 1.57 days of age respectively, while their teeth emerged ~10 days later at 54.9 ± 1.62 days of age. Single cubs experienced delayed timing in ear, eye, and teeth development compared to other litter sizes. Sex was not an important variable across any of our cub metrics. These results suggest that ABB cubs likely compensate for their unusually small size at birth by displaying faster growth rates during the first 6 weeks of life. Postnatal development differences between ABBs and other carnivores likely relates to reproductive strategies allowing black bears to synchronize with their environment and minimize energy expenditure during fetal cub development while in hibernation.

Introduction

Newborn American black bears (ABBs) (*Ursus americanus*) are the product of exceptional gestational adaptations. After mating, the fertilized embryo experiences obligate delayed implantation for over 100 days (Hamlett 1935). Thereafter, in the late fall, embryos resume their developmental activity to complete a 60-day active gestational phase that times parturition with hibernation (Hellgren et al. 1991). During this active gestational phase, females seek a den and subsequently reduce their metabolic rates and physical activity, as well as endure anorexia, adipsia (i.e., appetite loss), and anuria (i.e., lack of urination) (Nelson et al. 1983, Hellgren et al. 1990, Tøien et al. 2011). Parturition in ABBs usually occurs from mid-January to mid-February (Spady et al. 2007). At this stage bears give birth to altricial cubs that have closed eyelids and ear canals, a very short hair, and are much smaller in relation to the mother's weight compared to other mammals (Leitch et al. 1959, Case 1978). In fact, at birth, ABBs weigh between 0.3 and 0.4% of the adult body weight (Oftedal et al. 1993). In contrast, other mammalian species exhibit much greater proportions of body weight at birth, such as: American beaver (*Castor canadensis*) 3.8%, human (*Homo sapiens*) 4 - 5%, sea otter (*Enhidra lutris*) 7%, and American porcupine (*Erethizon dorsatum*) 20% (Case 1978). Moreover, ABB cubs exhibit lower growth rates (e.g., grams gained per day) than other carnivores, such as canids (Case 1978).

Most altricial species, including eutherian mammals, are born with minimal sensorial development that includes closed eyelids, closed ear canals, and gums lacking teeth (Fox 1964). Timing at which these organs achieve postnatal functional development varies greatly across species (Bekoff and Jamieson 1975, Braastad and Heggelund 1984, Suárez et al. 2017, Van Cruchten et al. 2017). For instance, spotted hyenas (*Crocuta crocuta*) are born with a set of

emerged teeth, eyes and ears open and appear to be sensitive to sound stimuli, whereas, raccoons (*Procyon lotor*) open their eyes and show tooth eruption after 21 and 30 days after birth, respectively (Pournelle 1965, Ewer 1998).

Some advances in understanding postnatal cub development in ABBs have shown that sexual dimorphism is not evident in denning cubs of the year and that morphological characteristics such as hair length and cranium width are positively associated with the age of cubs (Godfrey 1996, Ryan 1997, Echols 2000, Bridges et al. 2002). Growth rates of ABB cubs have been estimated around 50 g/day over two sampling sessions during the denning period (Farley and Robbins 1995).

In general, however, most of the cub growth and development information available for the ABB remains anecdotal or circumstantial resulting in a lack of understanding of detailed cub growth patterns relative to their litter size and timing of landmarks of sensory development such as ear canal and eyelid opening, and teeth eruption (Matson 1954, Clarke et al. 1980, Rogers 1986, Gray et al. 2016). This lack of information is likely due to the difficulty in obtaining data associated with the denning nature of parturient females and their newborns in the wild (Bridges et al. 2002). Therefore, our objectives were to assess the effects of age, sex, and litter size on cub body weight (BW), daily weight gain (DWG), proportion of daily weight gain (PDGW), and determine temporal landmarks in sensory (ear and eye) and morphological (teeth) development from birth until a 3-4 weeks post den emergence (~12-14 weeks of age) and determine relationships between maternal food consumption post hibernation and cub growth.

Methods

Sampling Design

We included 129 cubs from 58 different litters (single cubs: n = 7, twins: n = 62, and triplets: n = 60) born from bears temporarily-held at Virginia Tech's Black Bear Research Center (VT-BBRC), as previously described in Hellgren et al. (1990). Free-ranging pregnant bears were captured by the Virginia Department of Inland Fisheries (VDGIF) in the mid-to-late summer of each year and released back into the wild with their cubs the subsequent spring. We provided adult females with dry dog food at ~170 kJ/kg/d between arrival and September 30th, we then increased the food from October 1st until December 7th, to 335-376 kJ/kg/d. Thereafter, we reduced the food available by 1-fold per week until the first week of January, when food was completely removed if the bear had not already stopped food consumption voluntarily. Food (~170 kJ/kg/d) was slowly reintroduced to bears arousing from hibernation at the end of March or beginning of April, or earlier if bears showed signs of hibernation arousal such as: defecating, increased physical activity, and water consumption. We offered water to bears *at libitum* during the extent of the study. We chemically immobilized adult females and obtained cub measurements every 10 days for ~14 weeks, from birth until den emergence (~mid-April) of each year, between 1988 and 2016. All procedures were previously approved by the Institutional Animal Care and Use Committee at Virginia Tech under protocols 98-069, 09-073, 12-112, and 15-162.

Birth Detection

The active gestation stage was closely monitored via transabdominal ultrasound (see Chapter 1). We implemented auditory and visual inspections of dens as pregnancies approached term (e.g., ~10 days before expected parturition). Inspections consisted of sessions that lasted

between 15 and 30 minutes twice a day (morning and afternoon), where cub cries and visual detection of cubs determined time of parturition.

Morphological and Litter Measures

While adult females were immobilized, we brought cubs to a climate-controlled room and measured cub BW (kg), DWG (g/d), age when ear canal and eyelids opened, and age when deciduous teeth erupted, as previously described in Bridges et al. (2002). We also estimated the proportional daily weight gain (PDWG, %), as the DWG divided by total BW, to correct for differences in cub weight that could mask possible differences in cub growth rates. We restrained cubs physically to attain BW measurements using spring scales (from 1988 to 2012; Pesola® Medio-Line ± 3% precision) or a pediatric digital scale (from 2013 to 2016; Brecknell USA, MS-20 ± 10 g sensitivity). Additionally, we collected information related to individuals and litters, such as: date of birth, sex (male or female), litter size (single, twin, triplet), date of maternal resumption of food consumption (i.e., hibernation arousal) and date of litter den emergence. We include dates for litter den emergence in only 6 of 58 litters (including 2 litters observed via remote video records, season 2016). Our direct visualization methods (from 1988-2015) and architectural design of our facilities limited us to document exacts dates for den emergence in the remaining 52 litters. We marked cubs by painting their nails with color-coded nail polish to identify individuals within the same litter in subsequent sampling.

Statistical Analysis

We used linear mixed modeling techniques (LMM) to explore whether the morphological response variables (BW, DWG, PDWG, age at ear canal and eyelid opening, and age at deciduous teeth eruption) had any association with explanatory factors such as, cub age

(structured in 10-day intervals), sex, and litter size. We used litter and cubs as random effects to account for repeated measures of individual cubs and within litters (i.e., individual mothers). We built statistical models for each morphological variable using all possible additive combinations of explanatory factors and two-way interactions. We performed model selection using Akaike Information Criterion (AIC) and we considered models as competing if $\Delta AIC \leq 2$. We obtained all estimates using the restricted maximum likelihood (REML) procedure from the top model; values are expressed as means ± standard errors, unless otherwise noted. We performed LMM in the lme4 package (V 1.1-13) implemented in the statistical software RStudio version 1.0.143, RStudio Inc. In addition, we used a one-way ANOVA to determine differences in timing when mothers resumed food consumption post hibernation.

Results

Cub Body Weights (BW)

We compared a total of 11 possible models to describe cub BW growth from birth until 3-4 weeks post den emergence. The interaction between cub age and litter size best explained cub BW patterns with no competing models (Table 3.1). Overall, we found that newborns (0-9 days old) averaged a BW of 0.443 ± 0.079 kg and increased on average over 9-fold to 4.084 ± 0.102 kg by 14 weeks of age (Fig. 3.1). Even though cubs were born with similar BW, an increasing difference in weights was evident across different litter sizes. Cubs born in twin litters were heavier overtime than litters of singles or triples (Fig. 3.1). In fact, BW differences were the largest between 50-59 days of age, when cubs from twin litters weighed on average 0.41 and 0.56 kg more than cubs from single and triplet litters, respectively. Thereafter (60-99 days), cubs in single litters gradually reached similar BW as twins, whereas, triplets continued to experience

lower BW than cubs from other litter sizes (Fig. 3.1). Based on these results we performed a *post hoc* quadratic regression between cub age and cub weight to provide growth curves by litter size for the ABB (Fig. 3.2).

Cub Daily Weight Gain (DWG)

We constructed a total of 11 possible models to describe cub DWG rates from birth until 3-4 weeks post den emergence. The interaction of cub age and litter size best explained cub DWG patterns with no competing models (Table 3.1). Overall, cubs displayed two different DWG rate patterns where cubs gained 30.6 ± 1.8 g/d from birth until 59 days of age, thereafter (60-99 days), cubs almost doubled DWG rates (57.3 ± 5.6 g/d). Twins experienced higher DWG rates (37.8 ± 1.8 g/d) than single (31.1 ± 1.7 g/d) and triplet (22.9 ± 0.7 g/d) cubs from birth until 59 days of age (Fig. 3.3). However, these differences in DWG were drastically decreased after 60 days of age, as single cubs surpassed DWG rates of twins by 19.3 ± 13.7 g/d and triplets exhibited similar DWG rates as twins (difference of 3.4 ± 6.4 g/d) (Fig. 3.3). Based on these results, we performed a *post hoc* analysis to explore differences in DWG between siblings (e.g., within litter variation of twins and triples) using model with cubs nested within a litter, mother, cub age, litter size, and the interaction between cub age and litter size. This model suggested that siblings within their litters did not experience different DWG (df = 66, $F = 0.954$, $P = 0.579$). However, there were significant differences in cub DWG across mothers (df = 50, F = 5.12, $P < 0.001$) and litter size, where of twins experienced more DWG than triples (df =1, $F = 14.16$, $P = 0.0002$), as previously described.

Cub Proportional Daily Weight Gain (PDWG)

We compared a total of 11 possible models to describe the PDWG from birth until 3-4 weeks post den emergence. Similar to DWG, the interaction of cub age and litter size best explained the PDWG displayed by cubs with no competing models (Table 3.1), however, the pattern was slightly different from DWG. In general, we observed higher PDWG from birth until 39 days of age, in which cubs experienced PDWG > 3.5% (e.g., faster growth phase). After 40 days of age, cubs tended to display somewhat stable PDWG rates between 2 and 3.5% (e.g., slower growth phase). The faster growth phase started with PDWG rates of 6.51 ± 0.25% and decreased by day 39 of age to 3.54 ± 0.21% (Fig. 3.4). During this period, triplets exhibited the lowest PDWG rates, 0.85% lower than cubs from single and twin litters. In comparison, cubs from single and twin litters showed similar PDGW during the early faster growth phase (4.02 ± 0.91% and 3.98 ± 0.89%, respectively). The slower growth phase (e.g., after 40 days of age) was characterized by different PDWG dynamics across different litter sizes. During this latter period, for instance, single cubs showed a higher PDWG (2.41 ± 0.25%), compared to triples (2.23 ± 0.12%) and twins (1.94 ± 0.2%). This trend was most evident after 70 days of age, when single cubs reached the largest PDWG during this latter slow growth phase (3.49 ± 0.82%), followed by triplets and twin cubs, respectively (2.19 ± 0.23% and 2.04 ± 0.29%) (Fig. 3.4). Based on these results, we performed a *post hoc* analysis to explore differences in PDWG between siblings (e.g., within litter variation of twins and triples) using model with cubs nested within a litter, mother, cub age, litter size, and the interaction between cub age and litter size. This model suggested that siblings within their litters did not experience different PDWG (df = 66, $F = 0.696$, $P = 0.964$). However, there were significant differences in cub PDWG across mothers (df = 50, F = 3.03, $P <$

0.001) and litter size, where of twins experienced more PDWG than triples (df =1, F = 9.49, P = 0.0022), as previously described.

Postnatal Ear, Eye, and Teeth Development

Opening of ear canal and eyelids was evident by direct observation of the ear canal and eyelid separation (Fig. 3.5A, Fig. 3.5B, and Supplementary Fig. 3.1). The iris of cubs retained a bluish coloration throughout the study (Supplementary Fig. 3.1). The progression of teeth eruption started with canines, followed by incisors and molars (Fig. 3.5C). We compared a total of 6 models to describe timing of ear canal and eyelid opening, and teeth eruption. The interaction of organ type (e.g., ear, eye, and teeth) and litter size best explained the timing of postnatal organ development displayed by cubs with no competing models (Table 3.1). Overall, cubs opened their ears and eyes simultaneously at 44.07 ± 1.84 and 44.63 ± 1.57 days of age, respectively, while deciduous teeth erupted approximately 10 days later (54.9 ± 1.62 days of age) (Fig. 3.2). Twin and triplet cubs exhibited ear canal and eyelid opening and teeth eruption at earlier ages than single cubs. On average, twins and triplets experienced ear canal opening 5.8 days earlier than single cubs (Fig. 3.5D). Twins and triplets also displayed eyelid opening 7.2 days earlier than single cubs (Fig. 3.5D). Lastly, the same trend in litter size was observed in deciduous tooth eruption, yet the timing difference was on average smaller at 3.9 days later for single cubs versus twins and triplets (Fig. 3.5D).

Onset of Food Consumption by Maternal Parent Post Hibernation

We determined the onset of the maternal food consumption post hibernation in 51 adult female bears, as the remaining 7 adult females and their litters were released into the wild right before the onset of food consumption. Mothers resumed food consumption at the same average

time regardless of litter size [singles: March 26th (95%CI = March 15th – April 7th); twins: March 30th (95%CI = March 27th - April 2nd); triplets: April 1st (95%CI = March 28th - April 2nd); $F_{2,48}$ = 0.3575, P = 0.701)], or irrespective of cub age (singles: 53.8 ± 5.7 d, twins: 58.4 ± 2.4 d, triplets: 59.1 ± 2.9 d; $F_{2,48}$ = 0.3535, P = 0.704). Regardless of litter size, mothers aroused from hibernation when cubs were 58.2 ± 1.8 days old (Fig 3.2).

Cub Age at Den Emergence

We are reporting cub age at den emergence in only 6 of 58 litters (including 2 litters observed via remote video records, season 2016) due to limitations in methods implemented from 1988-2015 (see methods section for details). Overall, cubs emerged at 65.3 ± 3.8 d of age and 8.5 ± 5.7 d after their mothers resumed food consumption (Fig. 3.2 and Supplementary Fig. 3.1).

Discussion

Life history plays an important role determining postnatal offspring development in eutherian mammals (Derrickson 1992). Pregnant ABBs experience hyperphagia in the fall to accrue adipose tissue to undergo hibernation, during which they experience a 2-month active gestational stage, parturition, and a ~2-month lactation period during winter, the time of lowest productivity in the Northern hemisphere (Hellgren et al. 1989, Spady et al. 2007). Our study provides evidence regarding postnatal cub development that aligns with the life history of ABBs: first, cubs are born very small relative to mother's body mass and under-developed as previously reported (Case 1978, Oftedal et al. 1993); second, cubs experience higher PDWG during their first month of life than at older ages to possibly account for underdevelopment at birth; third, sensorial organs, such as eyes and ears, and deciduous teeth eruption achieve full development

just before den emergence; and fourth, ABBs display maternal tradeoffs in litter size as cubs born in triplet litters gained less net and proportional weight per day during the hibernating period, but this trend was overturned after the maternal parent resumed food consumption after arousing from hibernation .

Interactions between cub age and litter size were consistently the best describers of ABB cub development throughout our models. Litter size has been known to influence offspring growth in other eutherian species. Similar to our results, offspring of the desert wood rat (*Neotoma lepida*) and wild rabbits (*Orictolagus cunicilus*) exhibit lower body weight as litter sizes increase (Myers and Poole 1963, Cameron 1973). These effects are likely related to nutritional resources provided by the maternal parent through milk. Even though it is known that nutritional quality of milk changes very little during the hibernating lactational period in the ABB (Oftedal et al. 1993), it is possible that the greater number of cubs (e.g., triplets) have to compete for similar milk volumes as twins or single cubs, thereby negatively impacting DGW and PDWG of triplets, which is reflected in lower net cub weight, as previously suggested for brown and polar bears (*Ursus maritimus*) (Robbins et al. 2012).

Single cubs tended to show slightly lower weights and DWG rates than twins, especially before maternal arousal from hibernation, but that trend was substantially reduced after the maternal parent resumed food consumption post hibernation. However, that is not the case in captive Japanese black bear cubs (*Ursus thibetanus japonicus*), where cubs born in litters of singles exhibit greater DWG than cubs born in litters of twins (32.7 vs 19.8 g/d, respectively), which is reflected in greater body weight of single cubs (Iibuchi et al. 2009). It is possible that maternal condition of ABB females producing single cubs is lower than those producing twins

(see maternal condition Chapter 3). Furthermore, we evidenced significant differences in cub DWG and PDWG across maternal parents producing twins and triplets. Hence, single and triplet cubs might be exposed to lower milk quality, or lower volumes, than cubs born in litters of twins, thereby limiting single cubs in achieving equal or greater DWG rates or similar times for organ development than twins. Another alternative is that single cubs exhibit lower rates of weight gain than twins or triplets because they do not stimulate milk production sufficiently as it has been shown in single rat pups compared to pups in larger litter sizes (Russel 1980). Cub DWG during the hibernating periods in our study was on average ~33% lower than previously reported for long-term captive ABB producing twin litters at the Bear Research Facility at Washington State University (Farley and Robbins 1995). Factors such as milk nutrient content and maternal body condition are associated with cub growth rates in brown bears (*Ursus arctos*) (Robbins et al. 2012). Thus, it is possible that long-term captive females in the Farley and Robbins (1995) study exhibited higher body conditions and provided better milk quality for cubs than our short-term, temporarily captive females.

Maternal energy intake can directly impact offspring DWG in mammals. For instance, lactating cheetah cubs (*Acinonyx jubatus*) experience increasing DWG rates as the maternal parent increases food consumption per day (Laurenson 1995). In our study, adult female bears aroused from hibernation and resumed food consumption at similar timing as previously reported in the wild (Johnson and Pelton 1980), when cubs were ~8 weeks old. At this stage, milk nutrient content increases in ABBs (Farley and Robbins 1995). Coincidentally, cub DWG gradually increased after adult females consumed food post hibernation regardless of litter size.

Despite bears displaying overall slower growth rates than other carnivores [e.g., domestic cat – *Felis catus* and domestic dog - *Canis lupus familiaris* (Widdowson 1965)], we documented a faster growth phase (e.g., higher PDWG) during the first 6 weeks of age than at older ages in our study. These results suggest that ABB cubs likely compensate for small size and low levels of organ maturity, including low body fat content at birth (Oftedal et al. 1993), by displaying their fastest growth rates during the first weeks of life.

Altricial species experience postnatal development of sensorial organs such as eyes and ears due to immaturity in organ organization and muscular and neural control (Van Cruchten et al. 2017). The ABB experiences delayed timing in ear canal and eyelid opening, and in tooth eruption compared to other carnivores. For instance, spotted hyenas are born with a set of emerged teeth, eyes and ears open and appear to be sensitive to sound stimuli, whereas, some canids like coyotes (C*anis latrans*) undergo deciduous teeth eruption at ~10 days old, followed by eyelid opening ~5 days later (Pournelle 1965, Snow 1967, Bekoff and Jamieson 1975). However, fishers (*Martes pennanti*), a species also experiencing delayed implantation, show a relatively delayed postnatal development of deciduous teeth (~40 days old) and simultaneous eye and ear openings (~48 days old) (Frost and Krohn 2005).

Even though domestic dogs and wolves (*Canis lupus lupus*) experience opening of eyes and ear canals around the same time (eyes ~13 and ears ~19 days old), both species display a 5-10 day delay to respond to visual and auditory stimuli (Bekoff and Jamieson 1975, Lord 2013). Despite the relative delay in postnatal eye and ear development in the ABB (e.g., ~44 days of age), cubs should have a ~21-day period to achieve complete functionality of these sensorial

organs when they emerge from the den (~65 days of age) and should be fully responsive to auditory and visual cues.

Sexual dimorphism is evident in adult ABBs (Bartareau et al. 2012, Pope et al. 2017). However, during the extent of our study, sex was not an important variable in postnatal development in ABB cubs, as previously noted (Godfrey 1996, Ryan 1997, Echols 2000, Bridges et al. 2002). It appears that energy allocated to reproduction and diet consumed at specific life stages may be influencing mammalian sexual dimorphism (Ralls 1977, Johnson et al. 2017, Pope et al. 2017). It is likely that ABB cubs do not display morphological sex differences throughout our study because they were exposed to similar food resources and were sexually immature. Sexual dimorphism in ABBs may begin as they consume different diets post den emergence and reach sexual maturity.

In summary, our results show that black bear reproductive strategies are synchronized with their environment and minimize energy expenditure during fetal cub development while in hibernation. Cubs are born relatively small and underdeveloped. In their first 6 weeks of age they grow at proportionally faster rates, yet litters of triplets experience lower weights, and lower DWG and PDWG rates. Thereafter, ear canals and eyelids open simultaneously (~ 6 weeks of age), followed by ~10-day delay in deciduous tooth eruption. At this stage, maternal parents resume food consumption and cubs increase their DWG, and differences in cub growth among litter sizes are drastically reduced.

Future directions

This study was cub- and litter- centered, yet there are several other aspects such as maternal effects and the environment that are likely to play an important role in development and

fitness (Lindström 1999). Therefore, understanding postnatal development of ABB cubs is a step forward for future studies aiming to explore factors related to climate, maternal body condition, and litter characteristics, which possibly affect birthing phenology and fitness of bears experiencing environmental change (Bronson 2009). Furthermore, our study provides important general knowledge on postnatal cub development that could improve fostering orphan cubs to surrogate females (Carney and Vaughan 1984, Benson and Chamberlain 2006).

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List of Tables and Figures

Table 3.1. Linear mixed modeling describing body weight (BW), daily weight gain (DWG), proportion daily weight gain (PDWG), and postnatal organ development of American black bear cubs from birth until 3-4 weeks post den emergence. Model variables include: age (10-day intervals from 0 to 99 days old), litter size (single, twin, or triplet), sex (male or female), a random effect for repeated measures on cubs (1|cubs), and a random effect for repeated measures within litters (1|litter). Additive models are represented with a plus sign (+) and interaction models with an asterisk (*). AIC: Akaike Information Criterion, ΔAIC: AIC difference from top model, Log(*l*): maximized log likelihood, K: number of estimable parameters, *w*i: Akaike weights.

Model Definition	AIC	ΔAIC	Log(*l*)	K	*wi*
Cub Body Weight (BW, kg)					
(1|Cub) + (1|Litter) + Age * Litter size	11739.2	0	-5837.6	29	0.9999
(1|Cub) + (1|Litter) + Age + Sex + Litter size	11867.1	127.9	-5917.6	16	0.0001
(1|Cub) + (1|Litter) + Age + Sex	11867.9	128.7	-5920	13	0.0000
(1|Cub) + (1|Litter) + Age + Litter size	11871	131.8	5920.5	14	0.0000
(1|Cub) + (1|Litter) + Age	11871.5	132.3	-5922.7	11	0.0000
(1|Cub) + (1|Litter) + Age * Sex	11880.8	141.6	-5917.4	20	0.0000
(1|Cub) + (1|Litter)	13521.8	1782.6	-6756.9	2	0.0000
(1|Cub) + (1|Litter) + Sex	13523	1783.8	-6756.5	4	0.0000
(1|Cub) + (1|Litter) + Litter size	13523	1783.8	-6755.5	5	0.0000
(1|Cub) + (1|Litter) + Litter size + Sex	13523.6	1784.4	-6754.8	7	0.0000
(1|Cub) + (1|Litter) + Litter size * Sex	13525.4	1786.2	-6754.7	8	0.0000
Cub Daily Weight Gain (DWG, g)					
(1|Cub) + (1|Litter) + Age * Litter size	5820	0	-2881	29	0.9999
(1|Cub) + (1|Litter) + Age + Sex + Litter size	5842.5	22.5	-2906.3	16	0.0001
(1|Cub) + (1|Litter) + Age + Litter size	5842.9	22.9	-2907.4	13	0.0000
(1|Cub) + (1|Litter) + Age	5860.4	40.4	-2918.2	11	0.0000
(1|Cub) + (1|Litter) + Age + Sex	5860.7	40.7	-2914.4	13	0.0000
(1|Cub) + (1|Litter) + Age * Sex	5873	53	-2915.5	20	0.0000
(1|Cub) + (1|Litter) + Litter size	6072.2	252.2	-3042.6	5	0.0000
(1|Cub) + (1|Litter)	6104.3	284.3	-3048.1	2	0.0000
(1|Cub) + (1|Litter) + Sex	6105.5	285.5	-3047.7	4	0.0000
(1|Cub) + (1|Litter) + Litter size + Sex	6127.2	307.2	-3057.6	7	0.0000
(1|Cub) + (1|Litter) + Litter size * Sex	6128.8	308.8	-3057.4	8	0.0000
Cub Proportional Daily Weight Gain (PDWG, %)					
(1|Cub) + (1|Litter) + Age * Litter size	2409.7	0	-1175.9	29	0.9996
(1|Cub) + (1|Litter) + Age + Litter size	2426.1	16.4	-1199	14	0.0003
(1|Cub) + (1|Litter) + Age + Sex + Litter size	2428.1	18.4	-1199	16	0.0001
(1|Cub) + (1|Litter) + Age	2434.4	24.7	-1205.2	11	0.0000
(1|Cub) + (1|Litter) + Age + Sex	2436.2	26.5	-1205.1	13	0.0000
(1|Cub) + (1|Litter) + Age * Sex	2438.6	28.9	-1198.3	20	0.0000
(1|Cub) + (1|Litter) + Litter size	2905.8	496.1	-1446.9	5	0.0000
(1|Cub) + (1|Litter) + Litter size + Sex	2907.8	498.1	-1446.9	7	0.0000
(1|Cub) + (1|Litter) + Litter size * Sex	2909.7	500	-1446.9	8	0.0000
(1|Cub) + (1|Litter)	2911.1	501.4	-1451.6	2	0.0000
(1|Cub) + (1|Litter) + Sex	2913.1	503.4	-1451.6	4	0.0000
Cub Postnatal Organ Development (Ear, Eye, and Teeth)					
(1|Litter) + Organ type * Litter size	994.8	0	-486.4	10	0.5660
(1|Litter) + Organ type + Litter size	997.3	2.5	-491.6	7	0.1621
(1|Litter) + Organ type	997.5	2.7	-493.8	4	0.1467
(1|Litter) + Organ type + Litter size + Sex	999.2	4.4	-491.6	9	0.0627
(1|Litter) + Organ type + Sex	999.5	4.7	-493.8	6	0.0540
(1|Litter) + Organ type * Sex	1003.2	8.4	-493.6	7	0.0085

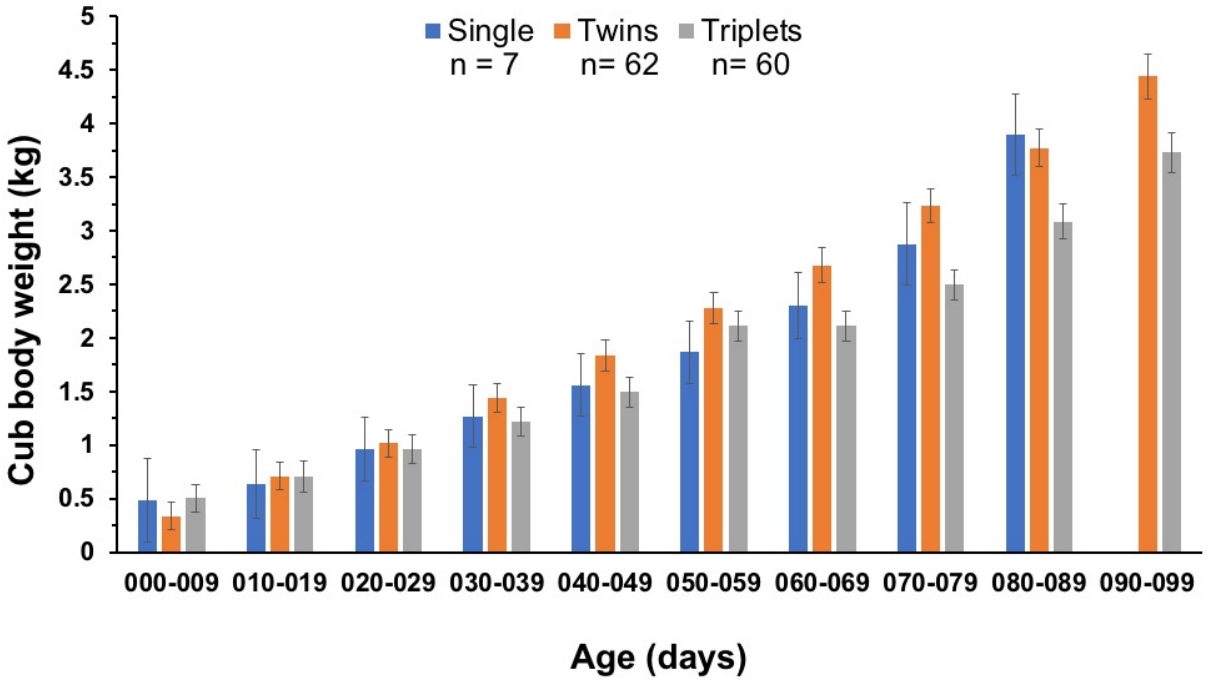


Figure 3.1. American black bear cub weights from birth until 3-4 weeks post den emergence. Estimates are from top statistical model: (1|Cub) + (1|Litter) + Age * Litter size (AIC=11739.2, *w*i = 0.9999, K = 29, error bars are 95%CI). We did not have data points from single cubs between 90 and 99 days of age.

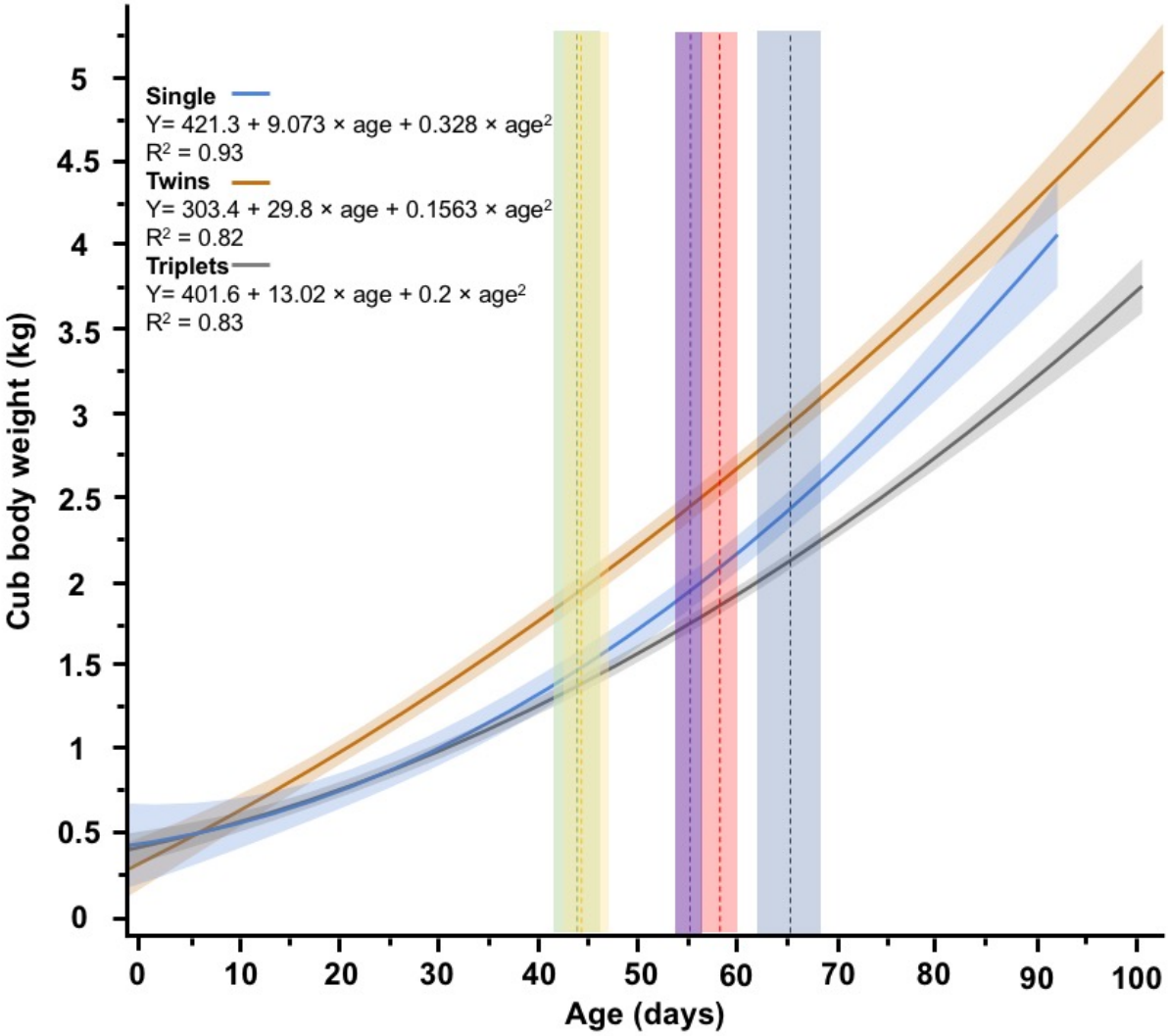


Figure 3.2. American black bear cub growth curves from birth until around 14 weeks of age. Curves are separated by litter sizes (single = blue, twins = orange, and triplets = gray). Shaded areas of the curve are 95% confidence intervals. Dashed vertical lines and illustrate the average and the width of vertical shaded areas represent SE for milestone timeframes for all cubs combined (e.g., regardless of litter size), such as: eyelid opening (yellow), ear canal opening (green) and, deciduous tooth eruption (purple), food consumption by maternal parent (red), after arousal from hibernation, and cub den emergence (gray-blue) (6 of 58 litters).

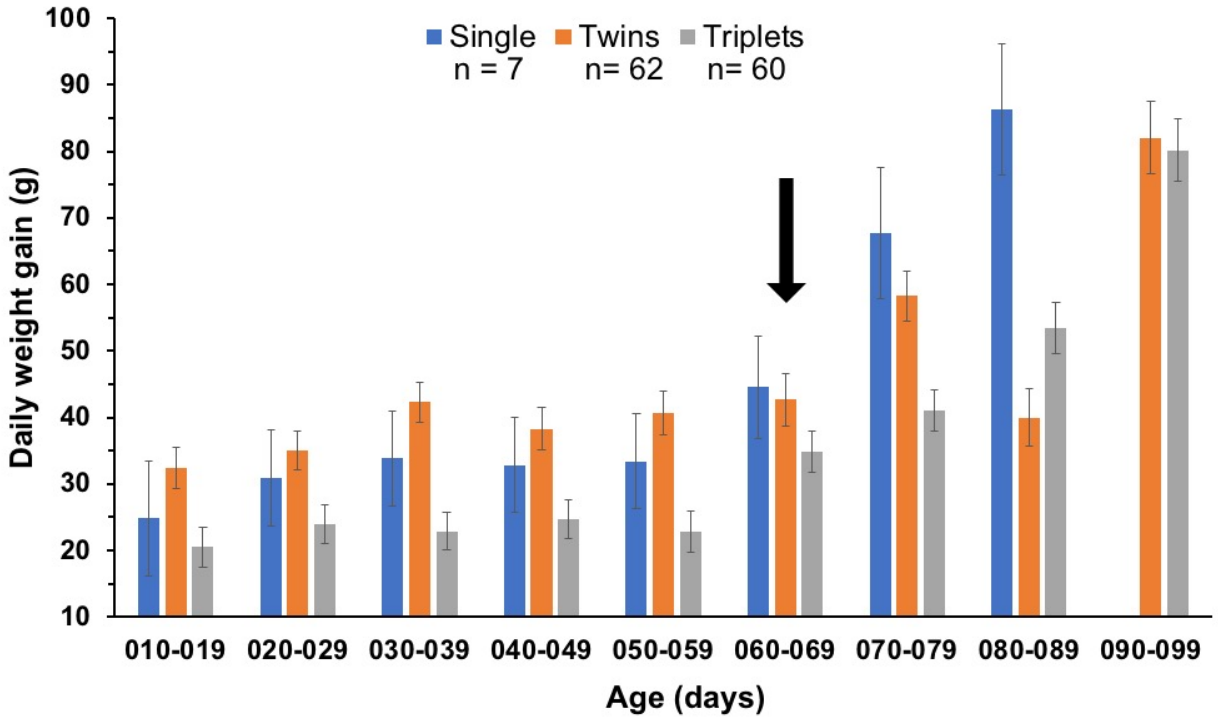


Figure 3.3 American black bear cub daily weight gain (DWG) from birth until 3-4 weeks post den emergence. Estimates are from top statistical model: (1|Cub) + (1|Litter) + Age * Litter size (AIC = 5820, *w*i = 0.9999, K = 29, error bars are SE). We did not have data points from single cubs between 90 and 99 days of age. Arrow showes average age of cub den emergence (n= 6 litters).

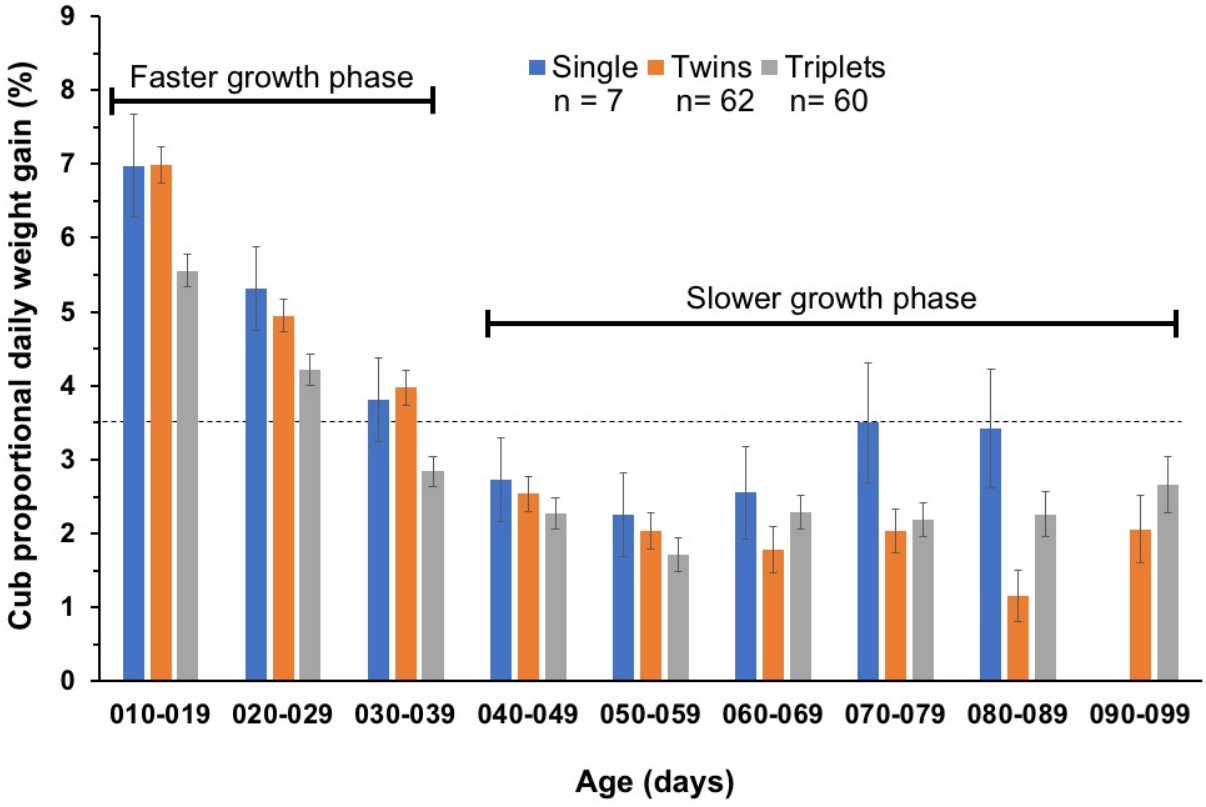


Figure 3.4. Average proportional daily weight gain (PDWG) of America black bear cubs. Cubs displayed bimodal weight gain phases: a faster phase, from birth until 39 days of age, and a slower phase, after 40 days of age. Dotted line illustrates the PDWG mark (3.5%) that differentiates the two PDWG phases. Estimates are from top statistical model: (1|Cub) + (1|Litter) + Age * Litter size (AIC = 2409.7, *w*i = 0.9996, K = 28, error bars are SE).

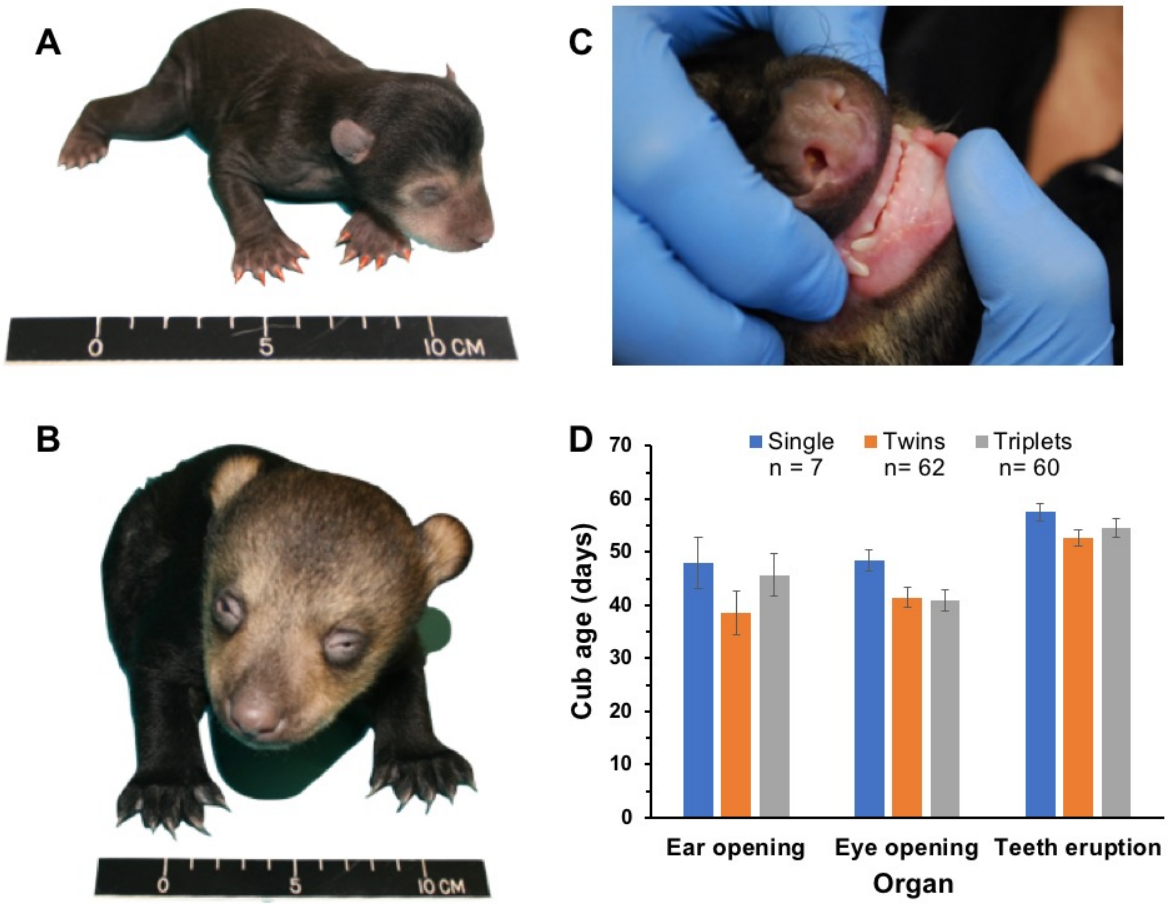
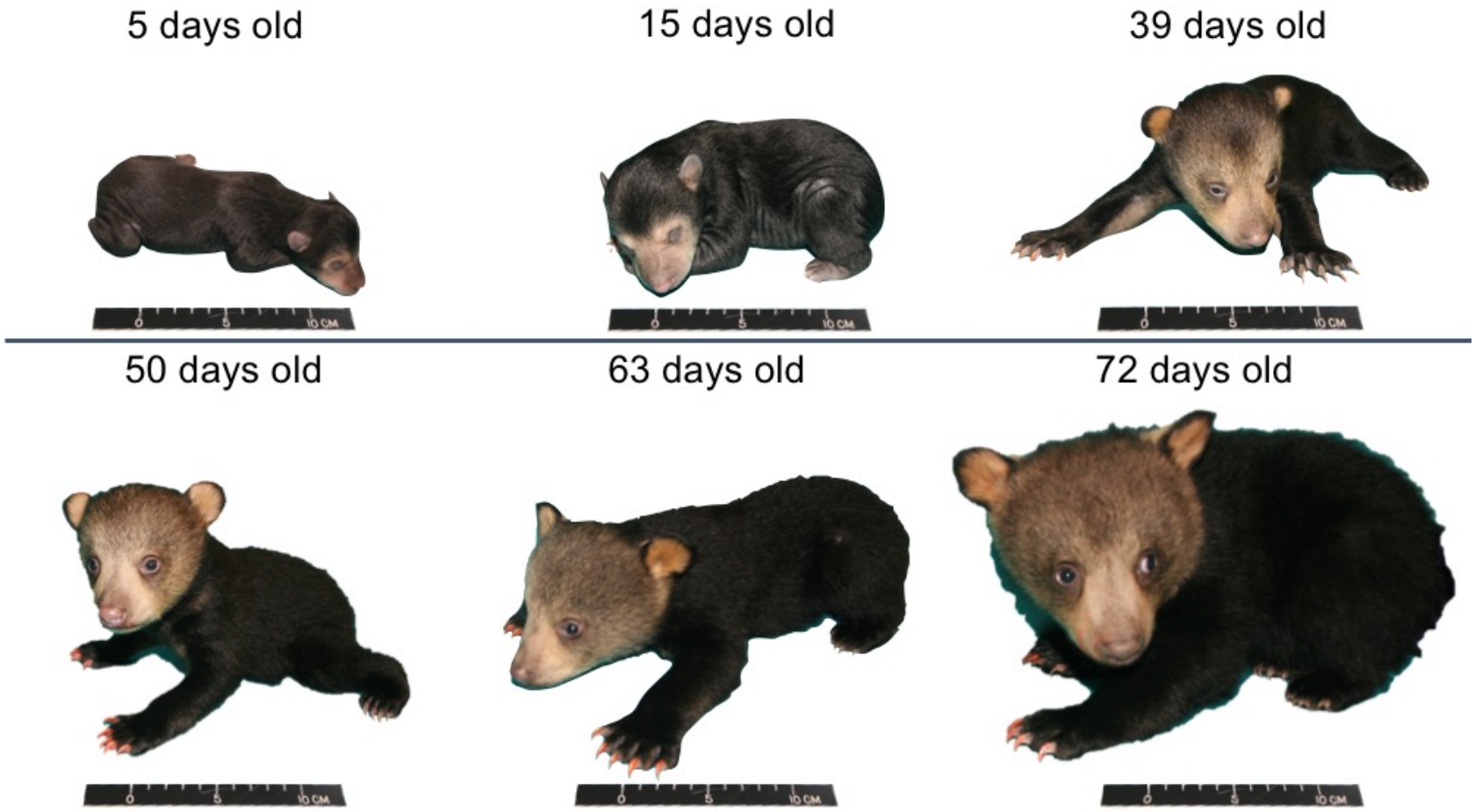


Figure 3.5. Timing of postnatal development of ears, eyes, and deciduous teeth of American black bear cubs. A. 5-day old cub with ear canal and eye lids closed; B. 39-day old cub starting to show separation of eye lids; C. 50-day old cub experiencing deciduous teeth eruption, canines emerged before incisors; D. Average age when cubs display opening of ear canals and eye lids, and deciduous teeth eruption, estimates are from top statistical model: (1|Litter) + Organ type * Litter size (AIC = 994.8, *w*i = 0.5660, K = 10, error bars are SE).



Supplementary Fig. 3.1. Morphological changes in a neonatal American black bear cub. This cub showed eyelid and ear opening at 39 days old and den emergence was observed after 63 days of age.

4. Chapter 3: Maternal Weight Dynamics in Fall and Hibernation Influence Embryonic Implantation Time, Litter Weight, and Cub Body Weight at Spring Hibernation Arousal in American Black Bears (*Ursus americanus*)

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Abstract

American black bears (ABBs) (*Ursus americanus*) display unique reproductive and hibernation physiological adaptations yet past in research on maternal effects on litter size and quality are conflicting. Understanding the role of maternal physical characteristics is important for bear management as environmental variation and habitat quality will impact adult bear body condition and reproduction, ultimately affecting bear population dynamics. The aim of this study was to determine the influence of maternal characteristics such as age and body condition, assessed through changes in maternal body weight, on prenatal (implantation timing, litter size) and postnatal effects (litter weight and cub body weight at birth and at spring arousal from hibernation) in temporarily captive ABBs giving birth at Virginia Tech's Black Bear Research Center. We used records from 48 adult females and 112 cubs born at our facility between 1988 and 2016. In our multivariate analysis, we included uncorrelated variables that were identified through stepwise selection procedures. The most important maternal variables included: maternal age, maternal body weight at onset of the hyperphagic phase (MBWHP), maternal daily weight gain from onset of hyperphagia until implantation in fall (MDWGF), and maternal daily weight loss from parturition until the end hibernation (MDWLEH). Important offspring variables influenced by maternal condition included: implantation day, litter size, cub body weight at birth (CBWB), cub body weight at the end of maternal hibernation (CBWEH), litter weight at birth (LWB), and litter weight at the end of maternal hibernation (LWEH). Our results provide evidence that maternal characteristics such as higher MBWHP and higher MDWGF are closely associated with earlier timing of embryonic implantation than in leaner females, which gained less weight per day in the fall. Females with higher MDWGF produced larger LWEH, whereas, females with greater MDWLEH were associated with greater CBWEH. Interestingly, CBWB was not associated with any maternal characteristic explored in our study, possibly due to the lower energetic cost of producing relatively small fetuses. Similarities in patterns and results obtained from our captive females with those of previous reports in free-ranging ABBs would allow future exploration of the effects of weather and climate variation on reproductive chronology and cub development, after accounting for maternal effects.

Introduction

Reproductive parameters of a population may be dictated by physiological and life history characteristics of a species, and/or by environmental conditions, and habitat suitability (Boyce 1992, Derrickson 1992, Lindström 1999, Loe et al. 2005, Rode et al. 2018). In particular, species that exhibit embryonic delayed implantation, a process by which fertilized embryos are arrested in their development until there is an optimal environment to carry a pregnancy, are an exemplary study species to explore how physiological adaptions allow for successful reproduction and offspring survival in changing environments (Sandell 1990, Mead 1993, Lopes et al. 2004, Mcallan and Geiser 2014).

The American black bear (ABB) (*Ursus americanus*) is among several mammal species that experience obligate delayed implantation (Spady et al. 2007). Mating occurs during late spring and early summer, a time when they consume high protein content foods (Hellgren et al. 1989, Spady et al. 2007). Thereafter, the delayed implantation period extends for over 100 days (summer until late fall), followed by reactivation of the embryo, subsequent implantation, and a 60-day active gestational phase during hibernation (Hamlett 1935, Hellgren et al. 1991). In the second half of the delayed implantation period, ABBs enter a hyperphagic state, in which they can increase their caloric intake over two fold by consuming foods with high caloric content in the fall (Hellgren et al. 1989). This hyperphagic phase allows bears to increase corporal fatty deposits in order to store energy supplies for overwintering (Nelson et al. 1983). Hibernation is characterized by anorexia, adipsia, and dramatic metabolic suppression in adult ABBs, yet pregnant females maintain their body temperature to foster an adequate environment for fetal development during the active gestational phase (Tøien et al. 2011, Chapter 1).

The study of maternal effects in the form of direct effects of non-genomic maternal

influences on offspring phenotype has been widely applied to mammalian species (Bernardo 1996). Due to the aforementioned reproductive and hibernation physiological adaptations, determining bear non-genomic maternal effects on litter size and cub quality, especially in a variable environment with variable habitat quality, will ultimately lead to better understanding of bear population dynamics. More specifically, bear maternal characteristics such as female body weight and body condition, have been shown to influence litter size, parturition chronology, and cub weights (Atkinson and Ramsay 1995, Samson and Hout 1995, Robbins et al. 2012). For instance, in Minnesota, ABB maternal body weight two months postpartum has a positive association with cub growth and litter weight (Noyce and Garshelis 1994). Adult ABB females losing on average 15.8 kg of body weight during the active gestation stage have been associated with producing twins, whereas females losing 19.8 kg produce 3 or more cubs in La Maurice National Park, Quebec (Samson and Hout 1995). However, neither maternal body weight in late December or two months postpartum are associated with litter size in ABBs (Noyce and Garshelis 1994, Samson and Hout 1995). Female polar bears (*Ursus maritimus*) at ages of 15 and 17 years produce significantly bigger single and twin cubs than any of the other females (5 – 27 years old) in the Hudson Bay (Derocher and Stirling 1998). ABBs in the Alleghany Mountains of Virginia tend to increase their litter sizes as they get older and synchronize their reproductive efforts with hard mast production, while younger females tend to give birth later in the year than older bears (Bridges et al. 2011*a*, *b*), yet age does not appear to influence time of parturition in brown bears (*Ursus arctos*) hibernating in Sweden (Friebe et al. 2014). However, associations of maternal body condition to implantation or parturition timing have not been reported in ABBs.

Even though body weight has been widely used as a proxy for body condition in ursids, different techniques assessing bear body condition have been developed to provide more

accurate metrics that account for differences in body size, body weight, and body composition across individuals. In fact, some of these techniques, including bioelectrical impedance analysis (BIA), isotope dilution, and a body condition index (BCI), have been widely used in ABBs, brown bears, and polar bears (Farley and Robbins 1994, Hilderbrand et al. 1998, Cattet et al. 2002). Interesting maternal effects have been elucidated using BIA in captive brown bears, where females with higher body fat content experienced parturition earlier in the year and their cubs grew at faster rates than leaner female bears (Robbins et al. 2012). Free-ranging female polar bears prior to den entrance experiencing higher body fat content, measured through isotope dilution, produced heavier cubs that were more likely to survive in the Hudson Bay area (Atkinson and Ramsay 1995). Similarly, female polar bears with higher corporal energy density, estimated through a BCI at den emergence, have higher probabilities to produce twins and triplets (Molnár et al. 2011).

Novel alternatives to assess bear body condition such as measuring body lipid content from fat biopsies and indirect estimates through measurements of serum leptin [a protein hormone associated with fat metabolism (Castracane and Henson 2006)] have been implemented due to logistic limitations in performing other techniques like BIA and isotope dilution in the field and/or acquiring specific body measurements for BCI (Spady et al. 2009, Mckinney et al. 2014, Sciullo et al. 2016). Even though fat biopsies have been used to monitor reproductive and non-reproductive body conditions of female polar bears in the Hudson Bay (Sciullo et al. 2016), very little work has been done to link serum leptin concentrations to maternal effects in ursids (Tsubota et al. 2008). Furthermore, been no previous reports of LWB or CBWB associated with maternal phenotypes in any ursid. Therefore, the aim of this study was to examine the influence of non-genomic maternal characteristics such as age and body condition, assessed through serum

leptin concentrations and changes in maternal body weight, on prenatal (implantation timing, litter size) and postnatal effects (litter weight and cub body weight at birth and at spring arousal from hibernation) in temporarily captive ABB females giving birth at Virginia Tech's Black Bear Research Center (VT-BBRC). The use of serum leptin as in indicator of ABB body condition could be especially useful at the VT-BBRC because this facility has an extensive serum bank that includes over 3,000 sera. This allows us to overcome some information deficiency in our long-term data set (e.g., over 25 years) because our data only includes BIA for 2 collection seasons, and total length measurements taken of bears at the VT-BBRC have been variable through time and did not follow those protocols of Cattet et al. (2002) to construct the validated BCI for ABBs.

Methods

In the late summer or early fall of each year, after the breeding season, free-ranging adult female black bears without cubs were captured from the wild by the Virginia Department of Game and Inland Fisheries (VDGIF) and temporarily housed at the VT-BBRC (17S 549889.34E 4118392N) between 1989 and 2016, as previously described in Hellgren et al. (1990). We provided adult females with dry dog food at ~170 kJ/kg/d between arrival and September 30th, we then increased the food from October 1st until December 7th, 335-376 kJ/kg/d. Thereafter, we reduced the food available by 1-fold per week until the first week of January, when food was completely removed if the bear had not stopped food consumption voluntarily. Food (~170 kJ/kg/d) was slowly reintroduced to bears arousing from hibernation at the end of March or beginning of April, or earlier if bears showed signs of hibernation arousal such as: defecating, increased physical activity, and water consumption. We offered water to bears *at libitum* during the extent of the study. We obtained cub measurements every 10 days for about 14 weeks, from

birth until den emergence (~mid-April) of each year, between 1988 and 2016. All procedures were previously approved by the Institutional Animal Care and Use Committee at Virginia Tech under protocols 98-069, 09-073, 12-112, and 15-162.

Sample Size

We included 4 females, median age 8 y (Min = 6; Max = 18), giving birth to single cubs, 24 females, median age 9 y (Min = 2; Max = 20), giving birth to twins, and 20 females, median age 7 y (Min = 3; Max = 15), giving birth to triplets. In total we used data from 48 adult females and 112 cubs born at our facility.

Obtaining Maternal and Cub Information

We chemically restrained females every 10 days with an intramuscular administration of a ketamine (4 - 8 mg/kg), xylazine (0.8 - 2 mg/kg), and occasionally a Telazol® (1.5 mg/kg) mixture to obtain blood samples and body measurements, including body weight (kg) using either hanging spring scales or a digital scale (Salter Brecknell VD 1000, MN, USA), from October until the subsequent spring (e.g., May). We also monitored pregnancies via transabdominal B-mode ultrasound (Aloka ecocamera SSD-500V and a 3.5MHz prove Hitachi-Aloka Medical America Inc.) from November to February. Dental annuli analysis was used to age females (Willey 1974) by Matson's Laboratory LLC (MN, USA)..

Embryonic implantation timing (e.g., surrounding time of embryo attachment to endometrium) was established by backtracking 60 days from parturition, as this is the suggested active pregnancy length in black bears (Hellgren et al. 1991).

We implemented auditory and visual inspections of dens as pregnancies approached term (e.g., ~10 days before expected parturition). Inspections consisted of sessions that lasted between 15 and 30 minutes twice a day (morning and afternoon), where cub cries and visual detection of

cubs determined time of parturition. We also recorded physical activity, food and water consumption, and date of maternal resumption of food consumption (i.e., hibernation arousal).

We restrained cubs physically to attain BW measurements using spring scales (from 1988 to 2012; Pesola® Medio-Line ± 3% precision) or a pediatric digital scale (from 2013 to 2016; Brecknell USA, MS-20 ± 10 g sensitivity), as previously described in Bridges et al. (2002). We estimated cub body weight at birth (CBWB, kg) using retroactive daily growth rates of cubs obtained in Chapter 2. Additionally, we collected information related to individuals and litters, such as: date of birth, litter size (single, twin, triplet), and estimated litter weight by adding weights from all cubs within the litter. We marked cubs by painting their nails with color-coded nail polish to identify individuals from the same litter.

Serum Leptin Measurements

We attempted to measure serum leptin using manufacturer directions of the multi-species leptin radioimmunoassay (RIA) (EMD Millipore Corp, MO, USA), which was previously reported for ABBs and brown bears (Hissa et al. 1998, Donahue et al. 2006). However, we performed immunoassay validations because the manufacturer recently changed the antibody stock and standard curves were dramatically different from previous versions of the RIA (see results section below). Therefore, we optimized the multi-species RIA by further diluting the provided antibody (1:3) with provided assay buffer and adding an extra standard (100 ng/mL, provided by manufacturer) to increase sensitivity and improve the shape of the standard curve (see results section below). In addition, we attempted immunoassay validations in serum leptin using four different commercially available ELISA kits; assays were performed as directed by manufacturers (Table 4.1). Immunoassay validations for ABB leptin included a linearity test and

accuracy recovery assessment for each assay, as described for other hormone kits by Mesa et al. (2014) (Table 4.1).

Furthermore, we implemented serum treatments due to poor validation results in some immunoassays (Table 4.1 and results section below). Serum treatments included: 1) Serum ultracentrifugation (10,000 *g* for 5 min, infranatant retrieved followed by a second ultracentrifugation cycle 10,000 *g* for 5 min) to remove lipids (Horney et al. 1999, Nikolac 2014, Saracevic et al. 2014); 2) Polyethylene glycol (PEG) precipitation by creating a 1:2 serum dilution with 25% (w/v) PEG-6000 (Fisher Scientific, USA) dissolved in phosphate buffered saline (PBS; 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH of 7.4) followed by a 10 min incubation at room temperature and a 10 min centrifugation (9,500 *g*) (Mccudden et al. 2010, Silva et al. 2014); and 3) A serum protein extraction protocol using trifluoroacetic acid and C18 Sep-Pack columns, following directions suggested for the ELISA oxytocin by ENZO Life Sciences, Inc.

Statistical Analyzes

We employed multiple linear regressions or analysis of variance (when appropriate) to determine the effect of maternal age (MA, y) and maternal weight metrics on implantation timing, litter size, cub body weight at birth (CBWB, kg), litter weight at birth (LWB, kg), and on cub body weight (CBWEH, kg) and litter weight (LWEH, kg) at the end of maternal hibernation, when mothers resumed food consumption in spring. Explanatory variables for implantation timing included: MA, maternal body weight at onset of hyperphagic phase in the fall (MBWHP), maternal body weight at time of implantation (MBWI, kg), maternal daily weight gain from onset of hyperphagic phase until implantation time in the fall (MDWGF, kg/d), proportional

maternal weight gain from onset of hyperphagic phase until implantation time in the fall (PMWGDF, %), and litter size.

Explanatory variables for litter size included: MA, maternal body weight at onset of hyperphagic phase in the fall (MBWHP), maternal body weight at time of implantation (MBWI, kg), maternal daily weight gain from onset of hyperphagic phase until implantation time in the fall (MDWGF, kg/d), proportional maternal weight gain from onset of hyperphagic phase until implantation time in the fall (PMWGDF, %), and implantation day.

Explanatory variables for CBWB and LWB included: MA, MBWF, MBWI, MDWGF, PMWGF, maternal body weight at parturition (MBWP, kg), and litter size. Explanatory variables for CBWEH and LWEH included: MA, MBWF, MBWP, MDWGF, PMWGF, MBWP, maternal body weight at end of hibernation (MBWEH, kg) maternal daily weight loss during pregnancy (MDWLP, kg/d), maternal daily weight loss from pregnancy until end of hibernation (MDWLPEH, kg/d), proportional maternal daily weight loss during pregnancy (PMWLP, %), proportional maternal daily weight loss during from pregnancy until end of hibernation (PMWLEH, %), and litter size.

We first screened explanatory variables for our models using correlation coefficients estimated by the restricted maximum likelihood (REML) method; if two variables were correlated (e.g., $r \geq 0.6$), we included the simpler and more intuitive variable and other was discarded from the analysis. The final model was constructed after further selecting explanatory variables using stepwise procedures with forward direction and the minimum Bayesian information criterion (BIC) as the stopping rule. All values are expressed as means ± standard

errors, unless otherwise noted. Statistical analyses were implemented in the software JMP® Pro (version 13.0.0, SAS Institute Inc.).

Results

Serum Leptin

We conducted an immunoassay validation for the EMD Millipore multi-species leptin RIA despite previous reports using this assay in ABBs (Donahue et al. 2006), as the kit manual (revised on June 17, 2016) reported a change in primary antibody (i.e., antibody recognizing bear leptin). In addition, we compared the standard curves for the previous antibody with the new antibody to test whether leptin concentrations of the new assay were similar to the previous antibody. We compared the curves by using with the curve produced by the new antibody obtained in 2016 and a representative curve retrieved from a former study in African elephants (*Loxodonta africana*) that used the previous antibody at our laboratory (e.g., Morfeld and Brown 2014). We found that the new assay has a standard curve that is dramatically different from the previous assay (Fig. 4.1). In fact, the new curve has a deficient shape, as it ranges only from 100 to ~45% binding and has a narrow flat-range (~50-80%), thereby limiting our ability to implement adequate quality control for the estimated dose 20 (ED_{20}) and hampering our ability to determine concentrations in an accurate fashion (Fig. 4.1). Moreover, the ED_{50} of the new curve is ~3 times higher than the ED_{50} of the previous curve (Fig. 4.1). Therefore, we decided to optimize the new assay, to create curve dynamics as close as possible to those provided by the previous antibody, following methods by Ciabattoni (1987). After testing 5 different combinations of antibody and tracer concentrations, we found that the best curve was achieved by diluting the new antibody (1:3), using the tracer at concentrations suggested by the manufacturer, and adding a 100 ng/mL standard to the curve (Fig. 4.1). Nevertheless, the optimized curve overestimated the ED_{50} by 2-fold compared to the previous

antibody (Fig. 4.1). In addition, recovery tests revealed poor accuracy of human leptin in bear sera (Min = 35.5 %, Average = 61.9 %, Max =101.5) (Table 4.1). Therefore, we treated the serum using different methods, including ultracentrifugation, PEG precipitation, and serum protein extraction, in attempts to remove possible sources of interference (see methods section for detail). Overall, these serum treatments slightly increased the recovery test accuracy, but they also removed the bear leptin present in the samples (Table 4.1).

Thereafter, we conducted validation tests for other immunoassays in leu of the suboptimal performance of the multi-species leptin RIA. We tested canine (EMD Millipore, MO, USA), human (R&D Systems, Inc., MN, USA), and porcine (Biomatik LLC, DE, USA) leptin ELISAS, following manufacturer directions, but tests were not satisfactory (Table 4.1). Unfortunately, porcine and canine immunoassays previously used to measure bear serum leptin were not available to us (Tsubota et al. 2008, Spady et al. 2009). Therefore, we were unable to include leptin analysis in this study.

Variable Selection

Our variable selection procedure found that MBWHP was highly correlated with MBWI (r = 0.8442), MBWP (r = 0.8115) and MBWEH (r = 0.7711), PMDWGF was highly correlated with MDWGF (r = 0.7728) as well as, PMDWLEH was highly correlated with MDWLEH (r = 0.8009). Therefore, we included MWHP, MDWGF and MDWLEH in the analyses.

Embryonic Implantation Timing

We included the following maternal variables: MA, MBWHP, MDWGF, and litter size to determine relationships with implantation time. The variable selection procedure identified MBWF and MDWGF as important variables related to implantation time. We constructed a

multiple linear regression model using MBWHP, MDWGF, and their interaction to assess their effect on implantation timing. This model was highly significant ($F_{3,44}$ = 11.73, P < 0.0001) and explained 44.4% of the variation. MBWHP had a significant negative relationship on time of implantation (t = -5.3, P < 0.0001), where heavier females with higher body weight at the onset of the hyperphagic phase in early fall (e.g., ~Oct 1st) were associated with implantation occurring earlier in the year (Fig. 4.2A). Similarly, females that gained more weight per day between the onset of the hyperphagic phase until the time of implantation experienced implantation significantly earlier in the year (t = -3.11, P = 0.0033) (Fig. 4.2B). The interaction between MBWHP and MDWGF did not have a significant effect on implantation timing (t = 1.25, P = 0.2173).

Litter Size

We included the following maternal variables: MA, MBWHP, MDWGF, and implantation time to determine relationships with litter size. The variable selection procedure identified MDWGF as the only important variable related to litter size. We constructed a logistic regression model to assess the effect of MDWGF on litter size. Females giving birth to triplets experienced, on average, higher daily weight gain (0.4 ± 0.02 kg/d) from early fall until implantation than females producing litters of twins (0.3 ± 0.03 kg/d) and singles (0.26 ± 0.1 kg/d) (Fig 4.3A); however, this relationship was marginally significant (df = 2, χ^2 = 5.58, P = 0.0614) (Fig. 4.3B).

Litter Weight and Cub Body Weight at Birth

We included the following maternal variables: MA, MBWHP, MDWGF, and litter size to determine relationships with LWB and CBWB. The variable selection procedure identified litter size as an important variable related to LWB. We constructed an ANOVA to test the effect of litter

size on LWB. LWB was significantly greater as litter size increased (R^2 = 0.252, $F_{2,2}$ = 7.58, P = 0.0015); LWB of triplets (0.926 ± 0.058 kg) were significantly heavier than LWB of singles (0.406 ± 0.13 kg) (Tukey HSD $P < 0.05$), but LWB of twins (735 ± 0.053 kg) were not significantly different from LWB of triplets and singles (Tukey HSD $P > 0.05$) (Fig. 4.4A).

In contrast, the variable selection procedure for CBWB did not identify any of the included variables as explanatory (i.e., the intercept was the best ranked model). In fact, we observed little difference in CBWB across litter sizes (Fig. 4.4B).

Litter Weight and Cub Body Weight at End of Maternal Hibernation

We included the following maternal variables: MA, MBWHP, MDWGF, MDWLEH, and litter size to determine relationships with LWEH and CBWEH. The variable selection procedure for LWEH identified MDWGF, MDWLEH, and litter size as important variables. We then constructed a multiple linear regression model using MDWGF, MDWLEH, litter size, a two-way interaction between MDWGF and MDWLEH, in addition to a two-way interaction between MDWLEH and litter size, to assess their effect on LWEH. This model was highly significant ($F_{5,35}$ = 13.96, $P < 0.0001$) and explained 66.6% of the variation. LWEH had a positive significant association with MDWGF (t = 2.85, P = 0.0075), such that adult females that gained more weight per day in the fall produced larger LWEH (Fig. 4.5A). Similarly, LWEH had a positive significant association with litter size (t = 2.14, P = 0.035) such that LWEH of triplets (5.05 ± 0.3 kg) and twins (5.1 ± 0.3 kg) were higher than singles (1.7 ± 0.6 kg) (Fig 4.5B). MDWLEH and the interactions were not significantly associated with LWEH (t = -0.06, P = 0.95, t = -1.30, P = 0.202, and t = 0.85, P = 0.403, respectively).

On the other hand, the variable selection procedure identified MDWLEH as the only explanatory of CBWEH. We then constructed a linear regression to assess the effect of MDWLEH

on CBWEH. A negative significant relationship between MDWLEH and CBWEH was evident (R^2 = 0.218, t = -3.30, P = 0.0021), where adult females that lost more weight from parturition until the end of hibernation produced cubs with larger body weight at the end of hibernation (Fig. 4.5C).

Discussion

Serum Leptin

We were unable to obtain reliable ABB serum leptin measurements from all five immunoassays tested in this study. Even though the multi-species leptin RIA showed linearity with bear leptin, the recovery tests were subpar, indicating that there is some degree of interference in the antibody-leptin interactions. One of the most common forms of interference in these cases is lipemia (high lipoprotein concentration in blood), where lipoproteins present in serum can block antibody binding sites (Nikolac 2014). High concentrations of lipoproteins (e.g., triglycerides and cholesterol) have been reported in sera and plasma collected through the hyperphagic phase and hibernation in the ABB (Hellgren et al. 1993, Lohuis et al. 2005). Preemptive measures such as, taking samples from fasted individuals, and sera treatments such as ultracentrifugation, PEG-precipitation, and protein extraction have been suggested to reduce lipemia in sera (Tate and Ward 2004, Schiettecatte et al. 2012, Nikolac 2014). However, reducing lipemia through fasting in ABBs has little effect on serum or plasma lipoprotein concentrations due to high adipose activity during the hyperphagic and hibernating stages (Hellgren et al. 1993, Lohuis et al. 2005). Even though, we evidenced removal of lipids (e.g., high volumes of supernatant for ultracentrifugation, or large pellet formation in PEG precipitation) after implementing serum treatments, and observed improvements in recovery tests, we also removed partially or completely the leptin present in samples, as shown in our linearity tests. Furthermore, poor curve dynamics shown by the new

multi-species RIA antibody hampered our ability to conduct accurate ABB leptin measurements and subsequent association to maternal effects influencing litter phenotypes.

Interference in immunoassays has been an underestimated problem (Ismail et al. 2002), particularly when assessing leptin in ursids. For instance, leptin has been measured in the ABB and Japanese black bear (*Ursus thibetanus japonicus*) using at least 2 immunoassays in each species, though no recovery tests have been reported for any of the assays (Donahue et al. 2006, Nakamura et al. 2008, Tsubota et al. 2008, Spady et al. 2009). More concerns arise as the range for plasma leptin concentrations measured via the multi-species RIA for brown bears has been reported between 1-4 ng/mL (Hissa et al. 1998) and 2.5 - 4.7 ng/mL in serum of ABBs throughout all activity stages, including hibernation (Donahue et al. 2006). However, using a porcine assay, Spady et al. (2009) reports serum leptin concentrations ranging between 0 and 22 ng/mL in the ABB. The Japanese black bear presents an even more extreme case as serum leptin from females in all metabolic states have been reported from 0-15 ng/mL (Tsubota et al. 2008) up to 50-320 ng/mL (Nakamura et al. 2008) using different canine ELISAS.

Regardless of these issues, we believe that leptin could be a great indicator of body condition in ursids. Future research could focus on developing and testing a specific assay for the ABB that uses an antibody raised against bear leptin. Alternatively, other sample purification methods could be employed, such as, treating serum samples with Lipoclear™ (Statspin™, HemoCue America) (Raff et al. 2003, Saracevic et al. 2014), or testing different PEG precipitations protocols using different centrifugation speeds or different PEG molecular weights (Polson and Ruiz-Bravo 1972). Lastly, mass spectrometry methodologies should be explored to assess ABB leptin, as they have been useful in other taxa presenting hormone assessment challenges (Prokop et al. 2014, Jedrychowski et al. 2015, Pompach et al. 2016).

Embryonic Implantation Timing

We provide the first evidence supporting that not only maternal weight at the beginning of the hyperphagic phase is associated with implantation time in the ABB, but also the maternal ability to assimilate food to accrue weight between the hyperphagic phase and implantation time. Our data indicates that heavier females and higher maternal weight-gain abilities between the hyperphagic phase and implantation time lead to earlier embryonic implantation and subsequently earlier birth than females with lower weights or lower weight gain. Similar trends have been shown in relation to maternal body fat content in captive brown bears and body condition of female badgers (*Meles meles*), which also experience delayed implantation (Woodroffe 1995, Robbins et al. 2012).

Relationships between maternal age parturition have been explored in free-ranging brown bears and ABB (Bridges et al. 2011*b*, Friebe et al. 2014). Although our results are only congruent with patterns observed in brown bears as age does not appear to be an important factor in either implantation or parturition timing (Friebe et al. 2014). On the other hand, young ABBs females (≤5 years old) gave birth later in the year in the Alleghany Mountains of Virginia (Bridges et al. 2011*b*). Young ABB females in that study could have experienced lower habitat quality and lower body condition, which are factors that could cause later birth dates but may not be directly related to age in female black bears (Bridges et al. 2011*b*).

Litter Size

None of the maternal characteristics tested in our study were significantly associated with litter size, albeit we found near significance of higher MDWGF producing larger litter sizes. Similar results were found in free-ranging ABBs, as maternal weight prior to hibernation was not associated with litter size (Noyce and Garshelis 1994, Samson and Hout 1995). However, female

polar bears with higher body energy density (estimated through body condition models) have larger litter sizes (Molnár et al. 2011). Thus, maternal body condition in the fall might be, in fact, associated with litter size in ABBs, though weight alone might not fully explain the actual body energy stores (Farley and Robbins 1994, Hilderbrand et al. 1998). It is also possible that due to embryonic diapause (which allows successful reproduction in highly seasonal environments), maternal factors of ABBs associated with the mating season influence the number of eggs ovulated and thus litter size (Spady et al. 2007, Mcallan and Geiser 2014). Maternal age was not an important variable explaining litter size patterns in our study, yet free-ranging ABB females older than 5 years produced larger litter sizes than younger females in Virginia (Bridges et al. 2011*b*). However, similar to implantation timing, younger ABB females in that free-range study could have experienced lower habitat quality and lower body condition (Bridges et al. 2011*b*).

Litter Weight and Cub Body Weight at Birth

To our knowledge there have been no previous reports of LWB or CBWB associated with maternal phenotypes in any ursid. Neither litter weight or cub body weight at birth were associated with any maternal characteristic in our study. Cubs from any litter size were born with similar body weights and litter weight increased linearly as the number of cubs increased, as expected. In contrast, domestic animals such as dogs (*Canis lupus familiaris*) and pigs (*Sus scrofa domesticus*) have shown a decrease in offspring weight at birth as litter sizes increase (Wilsman and van Sickle 1973, Dziuk 1992). It has been proposed that the reproductive strategy of species giving birth during hibernation, such as ursids, is energetically economic (Mcallan and Geiser 2014). It is possible that producing relatively small-sized offspring at birth (Oftedal et al. 1993) has negligent maternal costs and therefore cubs are born with similar weights regardless of the number of siblings.

Litter Weight and Cub Body Weight at the End of Hibernation

Litter weight at the end of ABB maternal hibernation increased as litter size and MDWGF increased, whereas heavier individual cubs were associated with females that lost more weight per day during the maternal hibernating period after parturition. These results align with previous studies that identified heavier females producing higher litter weights and heavier cubs in free-ranging ABBs, and females lose significantly more weight during hibernation as their litter size increases (Noyce and Garshelis 1994, Samson and Hout 1995). Likewise, female brown and polar bears with higher body fat content 90 days after parturition, produced higher litter weights (Robbins et al. 2012). Overall, these findings highlight the importance for female bears to synchronized with their environment to produce larger cubs that are possibly more fit to survive after den emergence (Noyce and Garshelis 1994).

In summary our results provide evidence that maternal traits such as weight and their ability to accrue mass in the fall are closely associated with timing of embryonic implantation and litter weight at the end of hibernation. Additionally, greater rates of maternal weight loss during lactation in hibernation are associated with greater cub weights. Interestingly, the weight of cubs at birth was not associated with any maternal characteristics explored in our study possibly due to the lower energetic cost of producing relatively small offspring at birth. Species experiencing embryonic diapause are considered to be more flexible in their ability to exploit resources more efficiently to ultimately produce more offspring or offspring with higher survival probabilities (Robbins et al. 2012). Hence, assessing maternal effects on reproductive parameters could be used to assess potential consequences of shifts in birth dates due to rapid anthropogenic changes in the environment, which, combined with environmental stochasticity, could potentially increase human-bear conflict or altered black bear population dynamics (positive or negatively impacting

human-bear conflict). More importantly, similarities in patterns and results obtained from our temporarily captive females housed at the VT-BBRC, with those previously reports on free-ranging ABBs, would allow us to explore the future the effects of weather and climate variability on reproductive chronology and cub development, after accounting for maternal effects.

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List of Figures and Tables

Table 4.1. Summary of immunoassay validation tests for American black bear leptin circulating in serum. *Serum protein extraction protocol suggested in the oxytocin ELISA by ENZO Life Sciences, Inc.

Immunoassay and Sample Type	Linearity Test	Accuracy Recovery Test
RIA Multi-Species Leptin		
XL-85K (EMD Millipore Corp., MO, USA)		
Serum	Parallel displacement ($F_{1,8}$ = 0.002, P = 0.960)	Min = 35.5%, Mean = 61.9%, Max = 101.5%
Serum ultracentrifuged (10,000 *g* x 5min twice)	Antibody reacted with one sample only	Min = 15.7%, Mean = 55.5%, Max = 86.2%
Serum precipitated (25% PEG-6000)	Antibody did not react with samples	Min = 58.8%, Mean = 76.8%, Max = 86.6%
Extracted serum*	Antibody did not react with samples	Min = 47.8%, Mean = 68.4%, Max = 79.3%
ELISA Canine Leptin		
EZCL-31K (EMD Millipore Corp., MO, USA)		
Serum	Parallel displacement ($F_{1,3}$ = 3.84, P = 0.1448)	Min = -95.6%, Mean = -21.5%, Max = 29.6%
Serum ultracentrifuged (10,000 *g* x 5min twice)	Antibody did not react with samples	Min = 39.3%, Mean = 43.1%, Max = 45.7%
ELISA Human Leptin		
Quantikine (R&D Systems, Inc., MN, USA)		
Serum	Antibody did not react with samples	Min = 73.6%, Mean = 96.6%, Max = 103.8%
ELISA Human Leptin		
DuoSeT (R&D Systems, Inc., MN, USA)		
Serum	Antibody did not react with samples	Not run
ELISA Porcine Leptin		
EKA01465 (Biomatik, LLC, DE, USA)		
Serum	Non parallel displacement ($F_{1,8}$ = 35.3, P < 0.001)	Min = 31.5%, Mean = 37.2%, Max = 51.8%

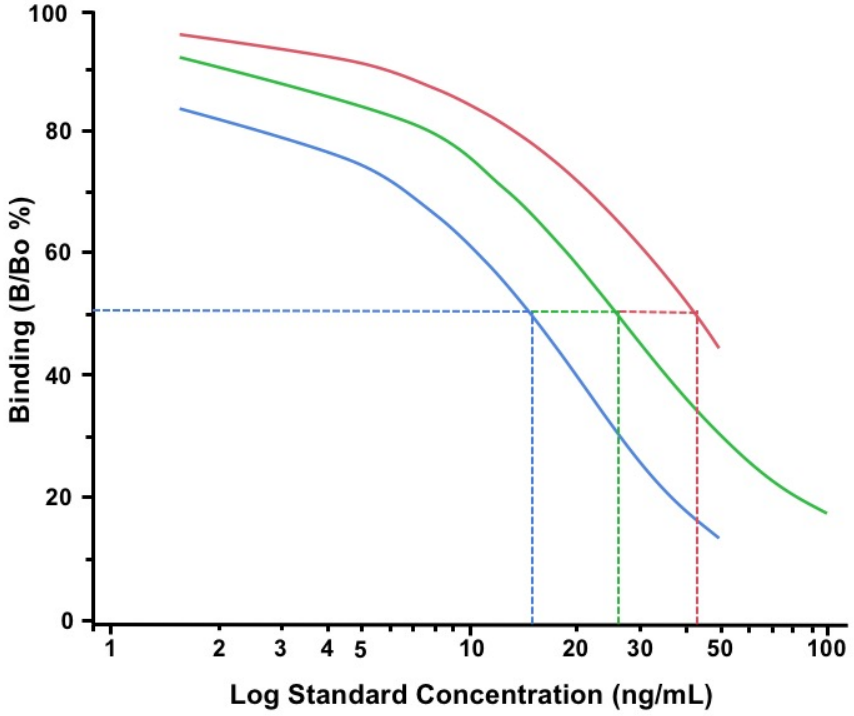

Figure 4.1. Comparison of different standard curves for the multi-species leptin RIA (MD Millipore Corp., MO, USA). Former antibody: blue; optimized current antibody: green; and current stock antibody: red. Solid lines represent standard curves and dotted lines represent estimated dose at 50% binding (ED50). Note the dramatic shift in ED50 from the former antibody to the current stock. In addition, the shape of the current antibody stock curve has a smaller region for accurate dose estimation (i.e., flat region of the curve).

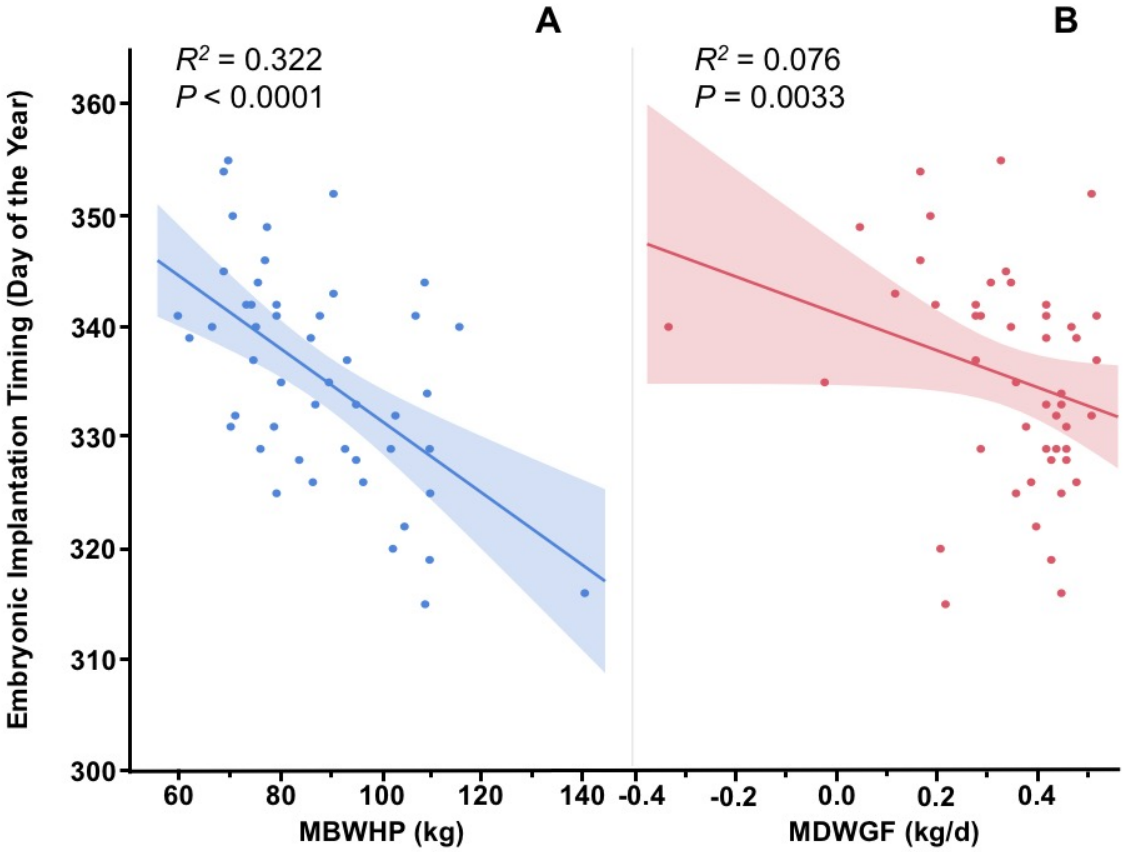

Figure 4.2. American black bear maternal characteristics associated with timing of embryonic implantation. **A.** Maternal body weight at onset of hyperphagic phase (MBWHP) displayed a negative, significant linear relationship with implantation day. **B.** Maternal daily weight gain in the fall (MDWGF), from onset of hyperphagic phase until implantation time, displayed a negative significant linear relationship with implantation day. Dots represent individual adult females and shaded areas are 95% confidence fits of the linear regression.

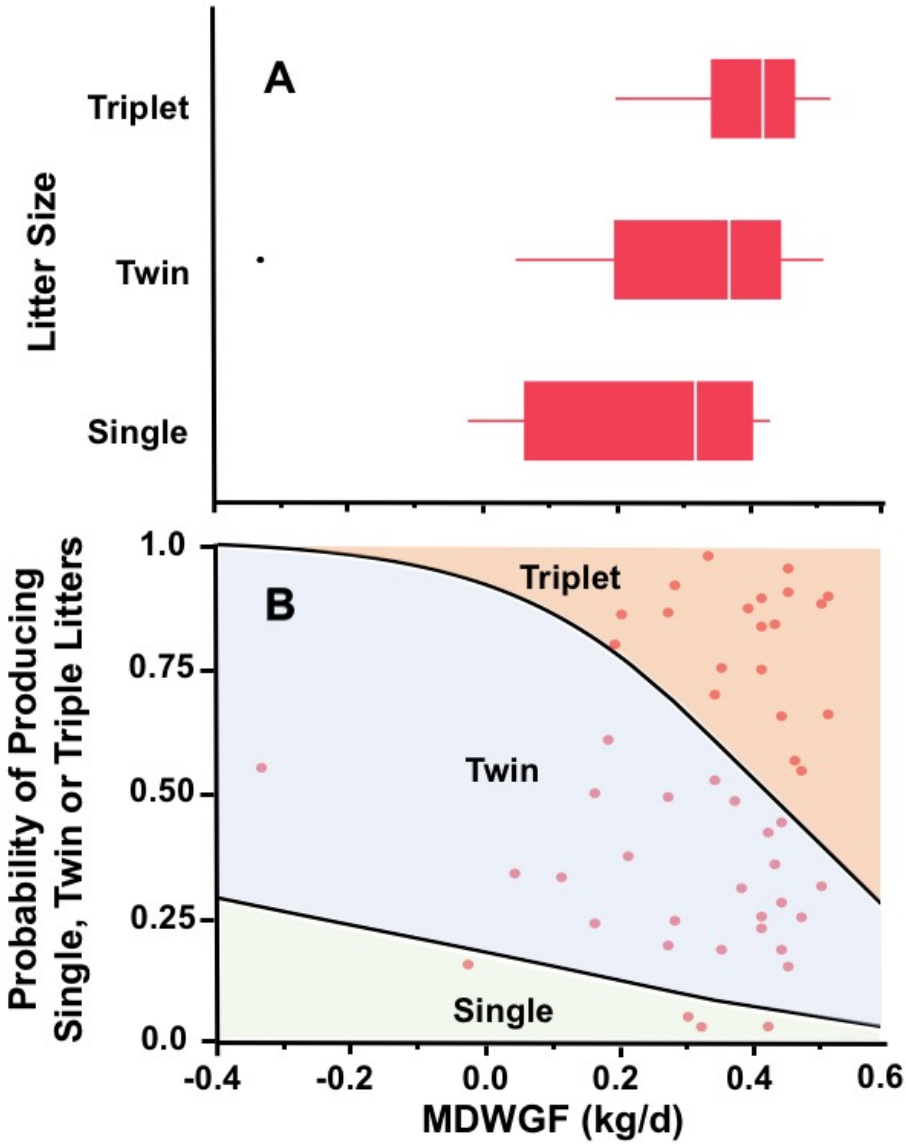

Figure 4.3. Relationship between litter size and maternal daily weight gain in the fall (MDWGF) from onset of hyperphagic phase until implantation time. **A.** Box and whisker plots of MDWGF distribution for females producing litters with single, twin, or triplet cubs (median: vertical white line through the box, confidence intervals, and outliers). **B.** Probability of producing different litter sizes given different MDWGF (single: green shaded area, twins: blue shaded area, triplets: orange shaded area, and individual mothers: red dots) (Logistic regression df = 1, $R^2 = 0.06$, $\chi^2 = 5.58$, $P = 0.0614$).

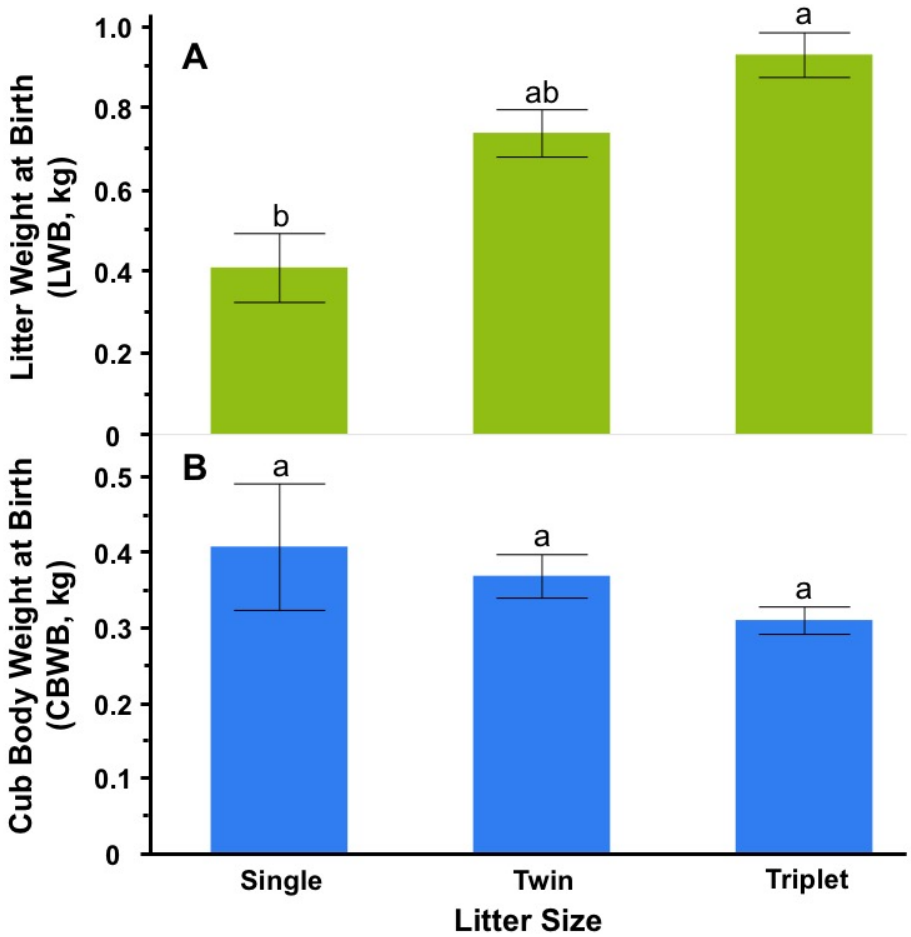

Figure 4.4. Litter size relationships to American black bear litter weight and cub weights at birth. **A.** Average litter weight at birth (LWB) increased significantly as litter size increased ($R^2 = 0.252$, $F_{2,2} = 7.58$, $P = 0.0015$). **B.** Average cub weight at birth (CBWB) had no association with litter size or any other maternal variable. No maternal variables were significantly associated with LWB or CBWB. Error bars are 1 standard error from the mean. Bars denoted with different letters are significantly different, Tukey's Honest Significant Difference (HSD) test ($\alpha = 0.05$).

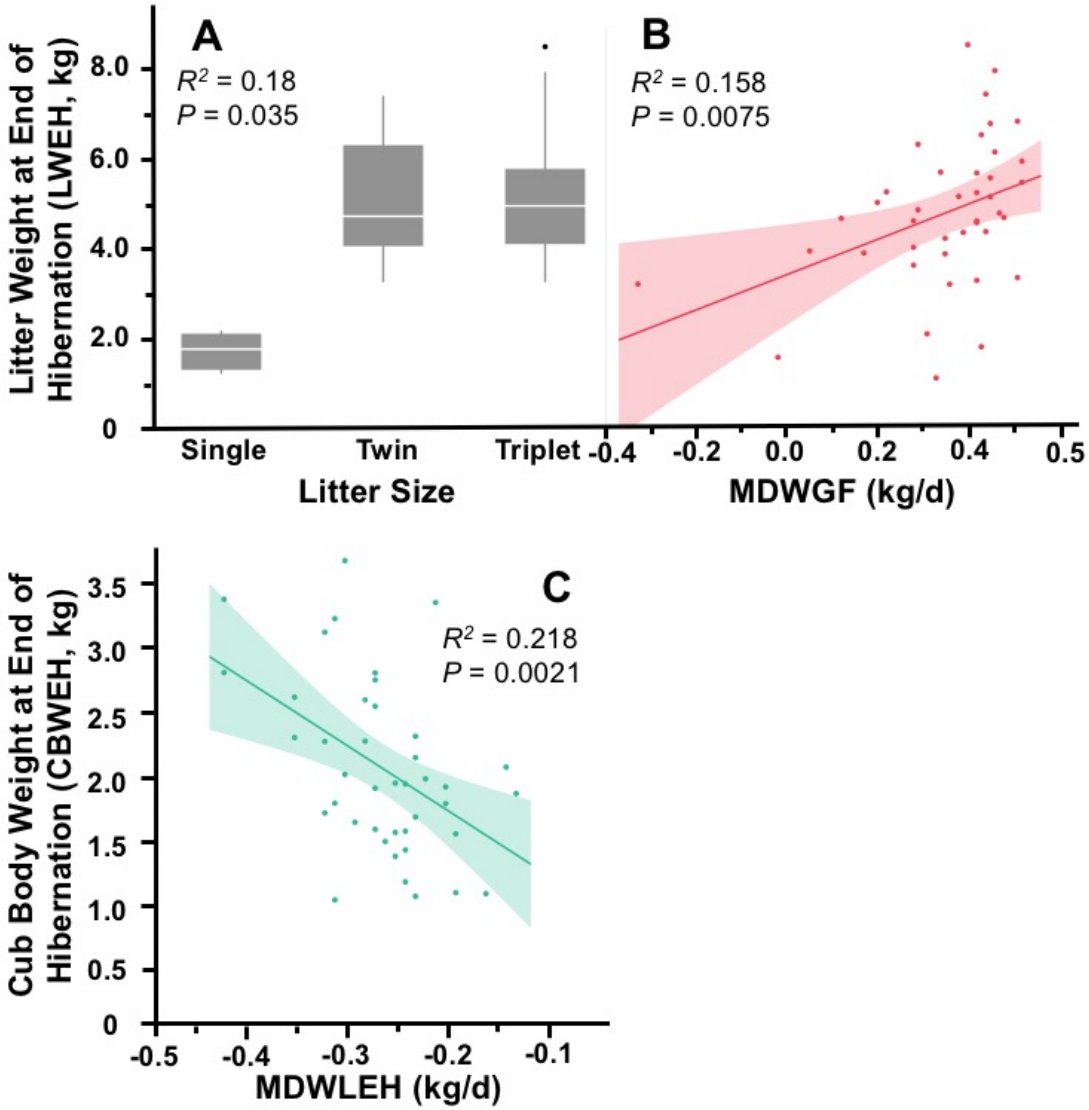

Figure 4.5. American black bear maternal characteristics associated with litter weight and cub body weight at the end of the maternal hibernation period. **A.** Litter weight at the end of maternal hibernation period (LWEH) was significantly higher in twins and triplets than in single cub litters. Box and whisker plots illustrating the median (white line through the box) confidence intervals, and outliers. **B.** Maternal daily weight gain in the fall (MDWGF), from onset of hyperphagic phase until implantation time, displayed a positive significant linear relationship with LWEH. **C.** Maternal daily weight loss from parturition until the end of hibernation (MDWLEH) had a negative significant linear relationship with cub weight at the end of maternal hibernation (CBWEH). Dots represent individual adult females and shaded areas are 95% confidence fits of the linear regression.

5. Chapter 4: Hibernating American Black Bears (*Ursus americanus*) Retain Skeletal Satellite Cell Myogenic Activity.

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Abstract

Bears, and other hibernators, are able to limit muscle atrophy during hibernation, a period of low metabolic rates, decreased physical activity, anorexia, and adipsia. Even though skeletal muscle functionality and architecture is regulated through multiple physiological pathways, the role of satellite cells (SCs), and their endocrine signaling, in hibernating species remain understudied. This study aimed to elucidate *in vitro* SC proliferation and differentiation associated with physical movement and serum myostatin in the American black bear (ABB) over different metabolic states, including fall hyperphagia, hibernation, and spring activity. We performed muscle biopsies and collected sera from adult males and females (n= 2 and 4, respectively) at Virginia Tech's Black Bear Research Center. Our results show that: 1) SCs maintain their ability to proliferate *in vitro* at similar rates throughout all ABB metabolic states, including hibernation, 2) *in vitro* SC differentiation and myogenic ability is increased during ABB hibernation, coinciding with a decrease in circulating serum myostatin, and 3) there are dramatically different nuclei fusion rates (i.e., differentiation) between males and females with cubs post hibernation, suggesting that reproductive females face additional metabolic constraints during spring arousal in order to maintain the integrity of skeletal muscle. We propose that maintaining the SC proliferative and differentiation abilities during hibernation is an important potential pathway for limiting muscle atrophy during bear hibernation. Future research could focus efforts on the role of torpor bouts, shivering, growth factors, and sex steroid hormones on SC dynamics during bear hibernation. Functional aspects of bear muscle conservation are interesting for understanding bear physiological adaptations to hibernation and also potentially for elucidating avenues to improve treatments for human metabolic disorders such as muscular dystrophy, sarcopenia, and disuse atrophy.

Introduction

The hibernating American black bear (ABB) (*Ursus americanus*) experiences physiological changes in the circulatory, respiratory, and digestive systems, and they decrease their basal metabolic rate by 75% to survive without ingesting any food or water over a 3-5 month period (Hellgren 1998, Tøien et al. 2011). Interestingly, some other systems, such as the musculoskeletal, exhibit little change during hibernation despite a drastic reduction in locomotor movement and a 4-8 °C decrease in body temperature (Tøien et al. 2015). Hibernating ABBs maintain most of their biomechanical skeletal muscle characteristics and only lose about half of the muscle strength that humans would lose under similar resting conditions (Lohuis et al. 2007*a*, *b*). In fact, evidence of ABBs avoiding skeletal muscle disuse atrophy during hibernation is plentiful (Koebel et al. 1991, Tinker et al. 1998, Lohuis et al. 2005, 2007*a*, *b*, Fedorov et al. 2009). Hence, hibernating bears possibly experience a suite of physiological adaptations for maintaining skeletal muscle function during hibernation, such as: shivering, regular neural activation, shifts in myocyte metabolic pathways, altered satellite cell (SC) activity, and/or increased endocrine modulation of muscle growth.

Shivering, uncoordinated muscle contraction, is used for hibernators to mediate thermoregulatory processes (Jastroch et al. 2016). Additionally, shivering during hibernation arousals in male greater tube-nose bat (*Murina leucogaster ognevi*), in complement with an increase in heat shock proteins, has been associated with muscle conservation during disuse (Lee et al. 2008). Shivering has been identified as a possible important adaptation for skeletal muscle conservation in the ABB, thirteen-lined ground squirrel (*Speromophilus tridecemlineatus*), and edible dormice (*Glis glis*) (Harlow et al. 2004, Malatesta et al. 2009, Tøien et al. 2015, Anderson et al. 2016), although, to date, no direct association between shivering and muscle conservation

has been determined in those species.

Regular neural activation of skeletal muscle has different effects in different activity periods in brown bears (*Ursus arctos*). During hibernation, unilateral denervation (e.g., surgical elimination of nerve function) of ankle flexor muscles, brown bears experience ~ 20% muscle mass reduction, whereas, during the summer active period, bears experienced a decrease in muscle mass three times greater in denervated than in intact muscles, as previously observed in other mammals (Lin et al. 2012). Therefore, neural stimuli appear important for other species in maintaining muscle mass, whereas in hibernating bears it has little effect on maintaining muscle mass and functionality during hibernation.

On the other hand, skeletal muscle biochemistry is relatively conserved during bear hibernation, although DNA concentrations increase and the protein:DNA ratio decreases during hibernation, while RNA concentrations increase significantly post hibernation (Koebel et al. 1991). More specifically, genes related to protein biosynthesis, such as RNA binding motif protein 3, ribosomal protein L37, and 40S ribosomal protein S23, increase expression activity in skeletal muscle of hibernating ABBs (Fedorov et al. 2009). Those changes indicate that there is potential for an increased capacity for skeletal muscle protein biosynthesis both during hibernation and after spring arousal to compensate for the minimal muscle fiber atrophy (10-20%), considering the 4-5 month period of fasting and inactivity (Fedorov et al. 2009).

Other aspects related to skeletal muscle conservation in hibernating mammals, including bears, remain understudied. Satellite cells (SCs) received their name based on their peripheral location in relation to skeletal muscle fibers (Mauro 1961). SCs are muscle stem cells with the capability to aid postnatal skeletal muscle growth and skeletal muscle regeneration in adults

through involvement in myonuclei recruitment to the myofiber (*i.e.*, differentiation), angiogenic processes, potential accumulation of intramuscular fat, and they regulate the muscle extracellular matrix (Dodson et al. 2010, Rhoads et al. 2013, Fry et al. 2017). Even though the understanding of the regulatory role of SCs in skeletal muscle is becoming more clear (Murach et al. 2018), we know very little of their role during mammalian hibernation. In fact, to our knowledge, SC activity in hibernators has been identified only in the thirteen-lined ground squirrels. During early torpor, these ground squirrels experience increased SC proliferation, which could explain the lack of muscle atrophy during hibernation (Brooks et al. 2015).

The endocrine regulation of skeletal muscle growth is, in part, influenced by myostatin, a transforming growth factor (a.k.a. TGF-8), which is a regulator of SC proliferation and differentiation in animals (Rhoads et al. 2009*a*). Mice and cattle, lacking myostatin, exhibit an increase in muscle mass due to a proliferation in myocyte number (i.e., hyperplasia) (Rhoads et al. 2009*a*). Mammals experiencing periods of skeletal muscle disuse or starvation tend to experience a rapid increase in myostatin, thereby decreasing the growth of skeletal muscle cells (Shao et al. 2007, Brooks et al. 2011). In contrast, thirteen-lined ground squirrels do not experience elevated expression of myostatin during hibernation. Interestingly, myostatin increases only after arousal from hibernation in ground squirrels, which contradicts the physiological pathways commonly found in non-hibernating mammals (Brooks et al. 2011). More recently, myostatin has been linked to myocyte metabolism in mice. Individuals lacking the myostatin gene tend to exhibit higher muscle mass but experience higher muscle fatigue, have myocytes that shift to a more anaerobic metabolism, and have decreased mitochondrial respiration (Mouisel et al. 2014).

It is clear that physiological strategies aimed at maintaining skeletal muscle function

during mammalian hibernation deviate from strategies used in non-hibernating species, yet the role of SCs and their endocrine signaling remain understudied. Therefore, the objective of this study was to elucidate SC proliferation and differentiation associated with physical movement and serum myostatin in the ABB during different metabolic states including hyperphagia [pre-hibernating (Pre-Hib)], hibernation (Hib), and spring activity [post-hibernating (Post-Hib)].

Methods

Animals and Husbandry

Adult ABBs (2 males: median age 2 years; and 4 females: median age 5 years) were temporarily housed at Virginia Tech's Black Bear Research Center (BBRC) in Blacksburg, Virginia (17S 549889.34E 4118392N), during two different sampling sessions 2013-2014 and 2014-2015. All females were pregnant and gave birth during hibernation. Cubs were raised by the females throughout the study. All procedures were approved by Virginia Tech's Institutional Animal Care and Use Committee (protocol numbers 12-112 FIW and 15-162 FWC).

The BBRC houses wild ABBs, brought by the Virginia Department of Game and Inland Fisheries, from late-summer until their release back into the wild in spring of the following year. Over this period, we housed bears individually and allowed acclimation to captive conditions, experiencing the hyperphagic state, hibernation, parturition, and resumption of the active state (Hellgren et al. 1990). We provided bears with commercial high-protein dry dog food (approx. 27% protein, 15% fat, 4% fiber; Pro Pet LLC, St. Marys, OH). We followed bear feeding protocols to mimic food consumption in the wild (Nelson et al. 1983, Hellgren et al. 1990). We offered ~170 kJ/kg/d from arrival until October 1st, then, we increased feed to 335-376 kJ/kg/d until November 30th. In December, we gradually reduced the feed by half per week and then completely removed it by around January 1, when bears entered hibernation. Finally, we offered

~170 kJ/kg/d after arousal from hibernation. Water was available *ad libitum* during the extent of the study, although water consumption during hibernation was not evident.

Sampling Design

We collected ABB serum samples every 10 days, from October to May, and conducted three muscle biopsies to capture three major metabolic states: hyperphagia (Pre-Hib), hibernating (Hib), and spring activity (Post-Hib), in October, February, and April, respectively.

Bear Chemical Restraint

We used one of two anesthetic drug combinations to facilitate sampling procedures. The first was a standard mixture of ketamine (4-8 mg/kg) and xylazine (0.8-1.6 mg/kg) and the second, a mix of Telazol® (1.5 mg/kg), ketamine (1.5 mg/kg), and xylazine (1.5 mg/kg). Both drug protocols were delivered with an intramuscular injection facilitated by an air pressurized dart-CO_2 pistol system (DanInject, DanWild LLC. Austin, TX). After bears were fully immobilized, we monitored vital signs every 5 minutes including heart and respiratory rates, and rectal temperature. Recovery was allowed to proceed without intervention if the bear showed signs of recovery at the end of the sampling procedure. Otherwise, we admistered a reversal agent (yohimbine 0.3 mg/kg S.Q., or P.R.) if anesthetic depth and climate conditions warranted.

Bear Aging

We removed the first upper premolar, a vestigial tooth of adult bears, with a sterile dental elevator. Bear ages were assessed through dental cemetum layer analysis (Willey 1974) by Matson's Laboratory LLC (MN, USA).

Locomotor Movement Quantification

We fit ABBs with a webbing collar (5 cm wide) containing an accelerometer sensor (+/- 29.4 m/s^2 HOBO pendant ® G logger, Onset Computer Corporation, Bourne, MA) around the neck to quantify movement. Gravitational forces (m/s^2) were measured every minute in three axes (suge, sway, heave). Locomotor movement is reported as the daily mean of the absolute sum of the diference between axial graviational forces (i.e.

$$\left(\frac{|(surge_t - surge_{t-1}) + (sway_t - sway_{t-1}) + (heave_t - heave_{t-1})|}{n_t}\right).$$

Serum Myostatin

We measured ABB serum myostatin using a commercially available ELISA (Quantikine®, R&D Systems, Inc. Minneapolis, MN). We conducted immunoassay validations through a linearity test (P = 0.7620) and accuracy recovery check (90.9 ± 3.7%), as described by Mesa et al. (2014). We performed procedures according to manufacturer instructions, including sample activation and immunoassay. Inter- and intra-assay variations were 4.5% and < 15%, respectively.

Muscle Biopsy

Myofiber samples originated from the *vastus lateralis* located on the lateral aspect of the posterior limbs (Lohuis et al. 2007 *b*). We attained biopsies from the left hind limb during the active (Pre-Hib and Post-Hib) states and from the right hind limb during the inactive state, Hib. We attained the third biopsy (*i.e.*, Post-Hib) by moving parallel to the femur in a straight line approximately 2.54 cm dorsally from the last biopsy site to avoid sampling scar tissue from the previous procedure. We prepared the biopsy area by clipping the hair and performing surgical disinfection. We made a skin insicion of about 1 cm in length. We obtained the biopsies with a 10-gauge x 9 cm long vacum assisted Vacora® Bard Biopsy Instrument (C.R. Bard Inc. Tempe, AZ)

inserted through the skin insition at a 45 degree angle to the orientation of the muscle fibers. Following the muscle biopsy extraction, we applied direct pressure to skin biopsy puncture site for hemostasis. We closed the skin insition with a topical tissue adhesive (Vetbobd™ 3M, St. Paul. MN). Lastly, we administered 2-3 mL of lidocaine (2%) subcutaneously around the insition edges, enrofloxacin (5 mg/kg, S.Q., S.I.D.), and flunixin meglumine (1 mg/kg, I.M., S.I.D.).

Satellite Cell Isolation

We isolated SCs from skeletal muscle (~250 g), as previously described by Rhoads et al. (2009*b*), with a minimal modifications. We removed connective tissue from muscle samples, then minced and digested the muscle tissue. We used differential centrifugation (1,500 *g*, 5 min) to separate SCs from muscle fiber fragments and tissue debris. SCs were pre-plated for 2 hours in pre-plating medium (Gibco™ DMEM 10% horse serum, 1% ABAM, and 0.5% gentamicin, Life Technologies, Carlsbad, CA). Thereafter, we collected cells in suspension, by centrifuging at 1,500 *g* for 5 min, and added them to 6-well plates coated with ECL media (Millipore, Billerica, MA) for growth.

In vitro Satellite Cell Proliferation and Differentiation

We induced cell proliferation and differentiation using previous methods with slight modifications (Rhoads et al. 2009*b*, Alexander et al. 2012). We seeded cells, in growth medium (MEM containing 20% FBS, 1% ABAM, and 0.5% gentamicin), at a density of $2.5X10^4/cm^2$. For proliferation, we seeded at density of 2000 cells/well in 96-well plates. We measured cell proliferation at 0 h, 24 h, 48 h, and 72 h using CyQuant ® NF proliferation kit (Life Technologies, Carlsbad, CA) following manufacturer protocol. We present proliferation as fold changes from time 0 h. For differentiation, cells were grown to 80% confluence and cell differentiation induced with differentiation medium (MEM containing 2% of horse serum, 1%

ABAM, and 0.5% gentamicin) for 4 days. At the end of differentiation, we subjected cells to immunocytochemical staining for myosin heavy chain (mf20 antibody was generously provided by Dr. Sally Johnson). Briefly, cells were fixed with 4% paraformaldehyde (Fisher Scientific, Fair Lawn, NJ) for 15 min and washed twice with ice-cold PBS. We incubated samples for 10 min with PBS containing 0.25% Triton X-100, and washed in PBS three times for 5 min. We then blocked samples with 1% BSA in PBST (0.5% Tween 20), and incubated with mf20 antibody overnight at 4 C. On the second day, we washed samples with PBS three times for 5 min, incubated with Dylight™ 488 secondary antibody (Fisher Scientific, Rockford, IL) for 1 h, and washed with PBS three times for 5 min. Lastly, we stained nuclei with 4′,6-diamidino-2-phenylindole (DAPI Sigma-Aldrich, St. Louis, MO) in PBS. We observed nuclei under an inverted microscope at 20X magnification. We counted ~ 1,000 nuclei for each sample and the fusion index is presented as percentage of nuclei within myotubes.

Statistical Analysis

We constructed linear mixed models (LMM) with the restricted maximum likelihood (REML) approach, accounting for repeated measures of the same individual over time (*i.e.*, random effect), to test whether locomotor movement, serum myostatin concentrations, and SC proliferation and differentiation, changed over three metabolic states (Pre-Hib, Hib, and Post-Hib). *Post hoc* Tukey HSD tests were employed when appropriate ($\alpha = 0.05$). Results are presented as means ± standard error of the mean (SE), unless otherwise noted. We used the statistical software JMP® PRO (version 13.0, SAS Institute Inc.) to perform analyses.

Results

Locomotor Movement Quantification

We report movement data on five of six individuals (2 males and 3 females) due to collar malfunction for one of the females. Bears exhibited highly significantly different levels of daily locomotor movement across metabolic states ($F_{2,782.5} = 164.2$, $P < 0.0001$). In fact, bears moved over 2-fold more Pre-Hib (0.9 ± 0.103 m/s^2) and Post-Hib (1.1 ± 0.102 m/s^2) than during Hib (0.47 ± 0.101 m/s^2) (Fig. 4.1).

Serum Myostatin

We analyzed 84 sera from the 6 individuals included in the study. Myostatin concentrations showed a highly significant difference across metabolic states ($F_{2,75.08} = 26.1$, $P < 0.0001$). Pre-Hib and Post-Hib periods exhibited similar myostatin concentrations (16.3 ± 1.67 vs 18.0 ± 1.68 ng/mL, respectively). However, during the Hib period bears experienced a significant decrease in myostatin concentrations (12.3 ng/mL ± 1.73 ng/mL) (Fig. 4.2A). Although our sample sizes precluded a quantitative comparison between sexes, males displayed overall slightly higher myostatin concentrations than females (18.9 ± 2.5 vs 13.9 ± 1.8 ng/mL, respectively), especially in the active periods (*i.e.,* Pre-Hib and Post-Hib) where the difference was more marked (Fig. 4.2A).

In vitro Satellite Cell Proliferation and Differentiation

In vitro SC proliferation rates were similar across metabolic states ($F_{2,41.7} = 2.04$, $P = 0.1427$). Proliferation significantly increased over incubation time ($F_{2,38.74} = 74.2$, $P < 0.0001$). Increments in proliferation by almost 2-fold were evident after 24 h, and continued to rapidly increase approximately 6-fold after 72 h of incubation (Fig. 4.3).

SC differentiation was confirmed by the formation of myosin-expressing myotubes using immunohistochemistry (Fig. 4.2C). SCs differentiated at similar rates over the metabolic stages ($F_{2,6}$ = 0.26, P = 0.2639). However, the overall nuclei fusion index was the greatest during hibernation (26.8 ± 4.4 %), whereas the Post-Hib (23.6 ± 4.4 %) and Pre-Hib states (22 ± 4.4 %) displayed overall lower differentiation rates (Fig. 4.2B). Although our sample sizes precluded a quantitative comparison between sexes, we observed a dramatic opposite trend between males and females in Post-Hib, where females exhibited the lowest ratio (11.6 ± 2.5 %) and males experienced 3-fold more nuclei fusion (35.6 ± 2.5 %). Interestingly, males tended to display slighly lower fusion index than females in the Pre-Hib and Hib states compared to Post-Hib (Fig. 4.2B).

Discussion

This study provides the first report of *in vitro* SC myogenic activity in hibernating bears and the first report of serum myostatin dynamics of ABBs during different metabolic states, including hibernation. Understanding the mechanisms by which skeletal muscle experiences low atrophy during periods of disuse has multiple applications to human and animal medicine (Stenvinkel et al. 2013, Doherty et al. 2014). Bears and other hibernators are able to limit muscle atrophy during hibernation, a period of low metabolic rates, decreased physical activity, anorexia, and adipsia (Nelson et al. 1983, Hellgren et al. 1990, Lee et al. 2008, Malatesta et al. 2009, Cotton and Harlow 2010). This study offers 3 main findings: 1) SCs maintain their ability to proliferate at similar rates throughout all ABB metabolic states, including hibernation; 2) SC differentiation and myogenic ability is maintained or slightly increased during ABB hibernation, coinciding with a decrease in serum myostatin; 3) There are dramatically different nuclei fusion

rates (i.e., differentiation) with males exhibiting higher rates than females with cubs Post-Hib, suggesting that females face additional metabolic constraints during spring arousal, likely to maintain the integrity of skeletal muscle.

During postnatal development, SCs facilitate skeletal muscle functionality by adding myonuclei to growing fibers or by forming new myofibers to replace injured muscle cells (Chen et al. 2017). The first step of this process is SC proliferation, which involves high regulation of proteoglycans and growth factors (Rhoads et al. 2009*a,* provides detailed information of these processes). We provide *in vitro* evidence that ABBs retain their SCs proliferative capacity at ~50 days into the hibernating state despite experiencing significantly lower locomotor movement and lack of food and water consumption. In contrast, humans undergoing relatively shorter periods of inactivity (14 days of bed rest), experience muscle atrophy by displaying significantly lower cross sectional areas, lower capillary density, and a decrease in SC proliferation (Arentson-Lantz et al. 2016).

Another hibernator, the thirteen-lined ground squirrel, has shown a significant increase of SC counts only during early torpor but not during late torpor or arousal periods (Brooks et al. 2015). It is unclear to us whether ABBs are able to maintain SC proliferation throughout the entire hibernating stage or if there are more specific dynamics in SC activity across torpor bouts within the hibernating stage. We were unable to track torpor bouts in our subjects, however, it is likely that the multiple individuals included in this study were at different torpor bout stages, since this research took place in two different sampling cohorts (years 2013-2014 and 2014-2015) and body temperature cycles in black bears can fluctuate in length from 1.6 - 7.3 days without circadian influence (Tøien et al. 2015). In addition, there is evidence that overexpression of protein biosynthesis genes in skeletal muscle of hibernating bears is maintained throughout

the hibernating period (Fedorov et al. 2009).

We observed a significant decrease in circulating serum myostatin in both males and females during hibernation, which coincided with a slight increase in SC differentiation and *in vitro* myogenic activity. Thereafter, circulating serum myostatin levels returned to pre-hibernating levels after spring arousal from hibernation. This finding suggests that ABBs are regulating SC activity during hibernation and that myostatin could influence SC proliferation and differentiation during bear hibernation. In contrast, torpid thirteen-lined ground squirrels exhibited similar myostatin expression as non-torpid individuals, possibly due to a decoupling of myostatin control over SC proliferation in early torpor (Brooks et al. 2011). In particular, myostatin expression only increased until arousal from hibernation in thirteen-lined ground squirrels. It is possible that ABBs use slightly different mechanisms to maintain skeletal muscle during hibernation than smaller hibernators. Interestingly, it appears that follistatin could be inhibiting myostatin activity in early torpor in thirteen-lined ground squirrels to allow SC proliferation in early torpor (Brooks et al. 2011, 2015). Future research could assess follistatin dynamics in ABBs to elucidate possible interactions between myostatin and SC activity throughout the different metabolic states.

Male bears tended to exhibit slightly higher circulating myostatin levels than females throughout the study. While we were not able to determine this difference statistically, this trend is usually reversed in species where males tend to have higher body and muscles masses than females, such as mice and humans (McMahon et al. 2003, Elliott et al. 2012).

An unexpected disparity in *in vitro* SC differentiation post hibernation was evident between sexes. Traditionally, it has been proposed that sex steroids influence muscle growth and

repair. However, the pathways by which sex steroids directly influence SC activity have been described only recently (Rhoads et al. 2009*a*, La Colla et al. 2015). For instance, estrogens can promote SC activation and proliferation through receptors α and β, and possibly influence subsequent SC differentiation, whereas androgens can directly stimulate SC differentiation through androgen receptor signaling (La Colla et al. 2015). It is possible that we observed sex differences in SC differentiation only during post hibernation due dramatic changes in sex steroid hormones that bears experience in spring after hibernation arousal. In our study, all females were pregnant and raised cubs. We have previously reported that pregnant bears experience a significant decrease in circulating 17β estradiol *post-partum* and maintain lower levels through initial stages of lactation (see Chapter 1). Additionally, males show a rapid significant increase in serum testosterone after arousal from hibernation to achieve active reproductive status in the summer (Tsubota et al. 1997). Future research is necessary to determine specific sex differences through interactions between hormone (myostatin, follistatin, and sex steroids) and their SC receptors and subsequent SC myogenic activity in reproductive and non-reproductive hibernating bears.

More recently, the immune system has been gaining more attention for its role in modulating SC activity in the skeletal muscle. Both the cellular (neutrophils macrophages) and humoral (cytokines, growth factors, and chemokines) immune responses to injured myotubes induce activation and proliferation of SC (Saini et al. 2016). Coincidentally, it has been reported that hibernating ABBs potentiate the presence of serum proteins associated with the immune system, including proteins of the acute phase, adaptive, and innate responses (Chow et al. 2013). Future research should explore the role of immune mediators in maintaining SC proliferation during bear hibernation.

In summary, functional aspects of bear muscle metabolism are interesting for understanding bear physiological adaptations to hibernation and for potentially elucidating avenues to better understand human metabolic disorders such as muscular dystrophy, sarcopenia, and disuse atrophy. We propose that maintaining the SC proliferative and differentiation ability during hibernation is an important potential pathway for limiting muscle atrophy during bear hibernation. Our research is a first step forward towards further exploring the intrinsic and extrinsic environments that influence SCs in remaining active during hibernation. Future research efforts should focus on determining the role of torpor bouts, shivering, and sex steroid hormones, such as estrogens and androgens, on SC dynamics during bear hibernation.

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List of Figures

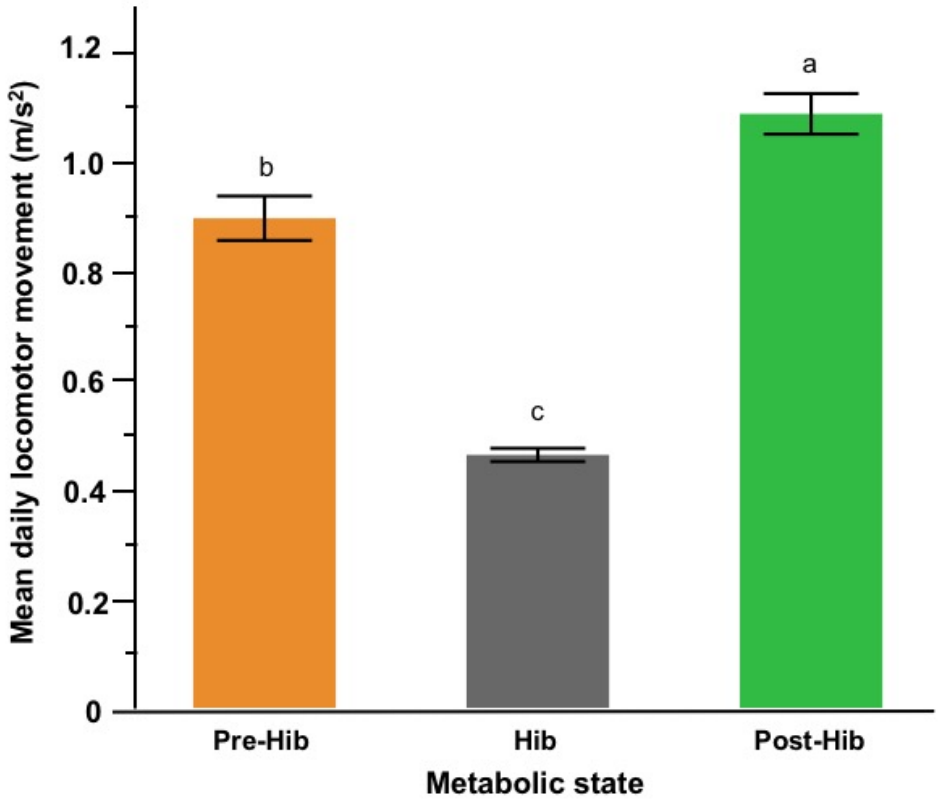

Figure 4.1. Mean daily movement, measured through tri-axial accelerometry in American black bears (*Ursus americanus*) experiencing different metabolic states from fall to subsequent spring: pre-hibernating (Pre-Hib), hibernating (Hib), and post-hibernating (Post-Hib). Error bars are 1 standard error from the mean. Bars denoted with different letters are significantly different, Tukey's Honest Significant Difference (HSD) test ($\alpha = 0.05$).

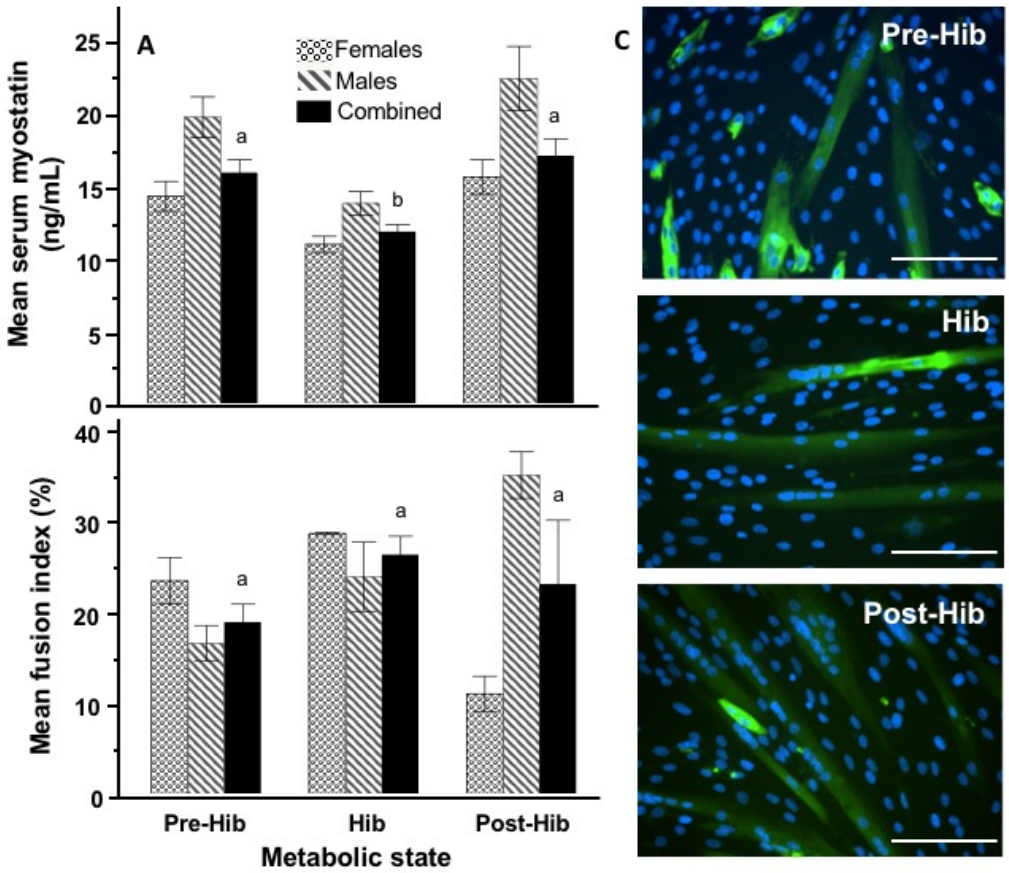

Figure 4.2. Hormonal and *in vitro* functional response of skeletal satellite cells (SCs) of American black bears (*Ursus americanus*) experiencing different metabolic states from fall to subsequent spring: pre-hibernating (Pre-Hib), hibernating (Hib), and post-hibernating (Post-Hib). A. Mean serum myostatin. B. Mean satellite cell fusion index (i.e., differentiation) expressed as the percentage of total nuclei located in myotubes after 4 days of *in vitro* incubation. Black and gray bars illustrate females and males, respectively. Striped bars illustrate the overall mean of combined sexes. Error bars are 1 standard error from the mean. Bars denoted with different letters are significantly different, Tukey's Honest Significant Difference (HSD) test (α = 0.05). C. Photomicrography of satellite cell-derived myotubes immunolabeled at day 4 of differentiation during each metabolic state; myotubes stained in green and nuclei stained in blue (Scale bar = 100μm).

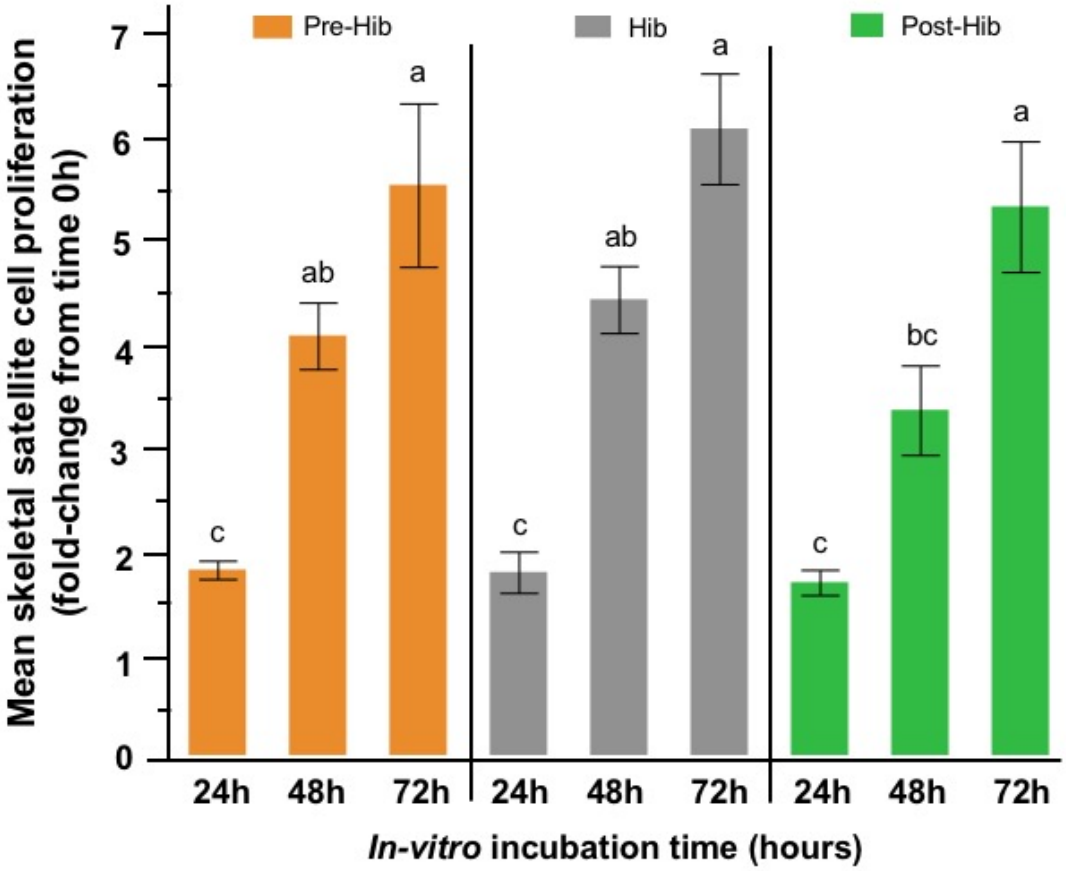

Figure 4.3. Mean skeletal satellite cell (SC) proliferation in American black bears (*Ursus americanus*) experiencing different metabolic states from fall to subsequent spring: pre-hibernating (Pre-Hib, orange), hibernating (Hib, gray), and post-hibernating (Post-Hib, green). SC proliferation is represented as fold changes from 0 hours of incubation. Proliferation was measured at 24, 48, and 72 hours of *in vitro* incubation. Error bars are 1 standard error from the mean. Bars denoted with different letters are significantly different, Tukey's Honest Significant Difference (HSD) test ($\alpha = 0.05$).

6. Conclusions and Future Directions

Contrary to previous studies, my study on pseudopregnancy provides little support for the exhibition of pseudopregnancy in free-ranging American black bears (ABBs). While I did find "pseudopregnant" serum progesterone (P_4) profiles in some bears that did not give birth, they were determined to have actually been pregnant during the active gestational stage, but later experienced *in utero* fetal loss, as has been previously been suggested (Hellgren et al. 1991). Taking these results and other reports of pseudopregnancy in hibernating bears into consideration, the occurrence of pseudopregnancy in free-ranging ABBs would require non-pregnant females that have ovulated to enter hibernation earlier in the year, maintain higher body temperatures, and use more energy from fat stores. Hence, there is no benefit for wild bears to exhibit pseudopregnancy during a period lacking energy resources to ultimately not attain the advantages of reproductive success. Pseudopregnancy in free-ranging ABBs is potentially less likely than in other non-hibernating bears, such as the Giant panda, because ABBs have different energy constrains during gestation and ABBs also have been recognized as metabolic marvels with highly adapted specialized metabolic strategies for using energy efficiently to increase reproductive success (Nelson et al. 1983, Stenvinkel et al. 2013). Therefore, contrary to previous notions, I provide evidence that pseudopregnancy might be rare or does not occur in free-ranging American black bear females.

Non-hibernating bears (e.g., Giant panda) might have slight physiological modifications in their reproductive cycles, but overall there is a major lack of understanding of reproductive physiology in these species. Therefore, it is possible that there are particular reproductive adaptations in some ursids but not in others, hence we should be cautious about extrapolating

physiological and behavioral reproductive strategies across ursids. Unfortunately, we know little about implantation mechanisms in ursids, so future exploration of the involvement of cytokines and acute phase inflammation proteins in maternal recognition and implantation could allow us to determine direct links between implantation and onset of hibernation in pregnant ABBs (Schäfer-Somi 2003, Kersey et al. 2016). Future studies could also determine the association of body condition with pregnancy loss in the ABB (Harlow et al. 2002, Spady et al. 2009).

While this dissertation increases our understanding of pregnancy physiology of ABBs, further research exploring direct causes of embryonic and fetal death, mechanism of *corpora lutea* reactivation in the fall, implantation mediators, mechanisms associated with metabolic regulation in hibernating pregnant females are warranted in the ABB.

Life history plays an important role determining postnatal offspring development in eutherian mammals (Derrickson 1992). Pregnant ABBs experience hyperphagia in the fall to accrue adipose tissue to undergo hibernation, during which they experience a 2-month active gestational stage, parturition, and a ~2-month lactation period during winter, the time of lowest productivity in the Northern hemisphere (Hellgren et al. 1989, Spady et al. 2007). My study on cub postnatal development provides evidence that aligns with the life history of ABBs: first, cubs are born very small relative to mother's body mass and under-developed as previously reported (Case 1978, Oftedal et al. 1993); second, cubs experience higher proportional daily weight gain (PDWG) during their first month of life to possibly account for underdevelopment at birth; third, sensorial organs, such as eyes and ears, and deciduous teeth eruption achieve full development just before den emergence; and fourth, ABBs display maternal tradeoffs in litter size as cubs born in triplet litters gained less net and proportional weight per day during the hibernating period, but this trend was overturned after the maternal parent resumed food consumption post

hibernation arousal. Despite the relative delay in postnatal eye and ear development in the ABB (e.g., ~44 days of age), cubs should have a ~21-day period to achieve complete functionality of these sensorial organs when they emerge from the den (~65 days of age) and should be fully responsive to auditory and visual cues.

Understanding postnatal development of ABB cubs is a step forward for future studies aiming to explore factors related to climate, maternal body condition, and litter characteristics, which possibly affect birthing phenology and fitness of bears experiencing environmental change (Bronson 2009). Furthermore, this dissertation provides important general knowledge on postnatal cub development that could improve fostering of orphan cubs to surrogate females with their own cubs (Carney and Vaughan 1984, Benson and Chamberlain 2006).

This dissertation provides evidence that maternal traits such as weight and ability to accrue mass in the fall are closely associated with timing of embryonic implantation, and litter mass at the end of hibernation, whereas, greater rates of maternal weight loss during lactation in hibernation are associated with greater cub weights. Interestingly, the weight of cubs at birth was not associated with any maternal characteristic explored in this study possibly due to the lower energetic cost of producing relatively small offspring at birth. Species experiencing embryonic diapause are considered more flexible in their ability to exploit resources more efficiently to ultimately produce more offspring or offspring with higher survival probabilities (Robbins et al. 2012). Hence, assessing non-genomic maternal effects on reproductive parameters could be used to assess potential consequences of shifts in birth dates due to anthropogenic changes in the environment, which combined with environmental stochasticity, could cause potentially increased human-bear conflict or altered black bear population dynamics (positive or negatively impacting human-bear conflict). More importantly, similarities in patterns and results obtained from our

temporarily captive females housed at the VT-BBRC with those previously reported for free-ranging ABBs would allow us to explore the effects of weather and climate variability on reproductive chronology and cub development, after accounting for maternal effects.

I was unable to obtain reliable ABB serum leptin measurements from five commercially available immunoassays. Regardless of these issues, I believe that leptin could be a great indicator of body condition in ursids. Future research should focus on developing and testing a specific assay for ABB that uses an antibody raised against bear leptin. Alternatively, other sample purification methods could be employed, such as, treating serum samples with Lipoclear™ (Statspin™, HemoCue America) (Raff et al. 2003, Saracevic et al. 2014), or testing different PEG precipitations protocols using different centrifugation speeds or different PEG molecular weights (Polson and Ruiz-Bravo 1972). Additionally, mass spectrometry methodologies should be explored to assess ABB leptin, as they have been useful in other taxa presenting hormone assessment challenges (Prokop et al. 2014, Jedrychowski et al. 2015, Pompach et al. 2016).

Lastly, this dissertation provides the first report of *in vitro* satellite cell (SC) myogenic activity in hibernating bears and the first report of serum myostatin dynamics of ABBs during different metabolic states, including hibernation. Understanding the mechanisms by which skeletal muscle experiences low atrophy during periods of disuse has multiple applications to human and animal medicine (Stenvinkel et al. 2013, Doherty et al. 2014). Bears, and other hibernators, are able to limit muscle atrophy during hibernation, a period of low metabolic rates, decreased physical activity, anorexia, and adipsia (Nelson et al. 1983, Hellgren et al. 1990, Lee et al. 2008, Malatesta et al. 2009, Cotton and Harlow 2010). This study offers 3 main findings: 1) SCs maintain their ability to proliferate at similar rates throughout all ABB metabolic states,

including hibernation; 2) SC differentiation and myogenic ability is slightly increased during ABB hibernation, coinciding with a decrease of serum myostatin; 3) There are dramatically different nuclei fusion rates (i.e., number of SCs that have differentiated into myocytes) with males exhibiting higher rates than females with cubs post hibernation, suggesting that females face additional metabolic constraints during spring arousal to maintain the integrity of skeletal muscle.

Functional aspects of bear muscle metabolism are interesting for understanding bear physiological adaptations to hibernation and for potentially elucidating avenues to better understand human metabolic disorders such as muscular dystrophy, sarcopenia, and disuse atrophy. I propose that maintaining the SC proliferative and differentiation ability during hibernation is an important potential pathway for limiting muscle atrophy during bear hibernation. This research is a first step forward towards further exploring the intrinsic and extrinsic environments that influence SCs in remaining active during hibernation. Future research efforts should focus on determining the role of torpor bouts, shivering, and sex steroid hormones, such as estrogens and androgens, on SC activity during bear hibernation.

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