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RESEARCH ARTICLE

Suppressed bone remodeling in black bears conserves energy and bone mass during hibernation

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ABSTRACT

Decreased physical activity in mammals increases bone turnover and uncouples bone formation from bone resorption, leading to hypercalcemia, hypercalcuria, bone loss and increased fracture risk. Black bears, however, are physically inactive for up to 6 months annually during hibernation without losing cortical or trabecular bone mass. Bears have been shown to preserve trabecular bone volume and architectural parameters and cortical bone strength, porosity and geometrical properties during hibernation. The mechanisms that prevent disuse osteoporosis in bears are unclear as previous studies using histological and serum markers of bone remodeling show conflicting results. However, previous studies used serum markers of bone remodeling that are known to accumulate with decreased renal function, which bears have during hibernation. Therefore, we measured serum bone remodeling markers (BSALP and TRACP) that do not accumulate with decreased renal function, in addition to the concentrations of serum calcium and hormones involved in regulating bone remodeling in hibernating and active bears. Bone resorption and formation markers were decreased during hibernation compared with when bears were physically active, and these findings were supported by histomorphometric analyses of bone biopsies. The serum concentration of cocaine and amphetamine regulated transcript (CART), a hormone known to reduce bone resorption, was 15-fold higher during hibernation. Serum calcium concentration was unchanged between hibernation and non-hibernation seasons. Suppressed and balanced bone resorption and formation in hibernating bears contributes to energy conservation, eucalcemia and the preservation of bone mass and strength, allowing bears to survive prolonged periods of extreme environmental conditions, nutritional deprivation and anuria.

KEY WORDS: Skeletal adaptation, *Ursus americanus*, CART, Calcium metabolism, Disuse

INTRODUCTION

Bones perform essential mechanical functions in vertebrates, and therefore bone mass is maintained in proportion to the mechanical loading the skeleton experiences. Bone loss due to physical inactivity is a universal feature in humans and the experimental menagerie (e.g. mice, rats, turkeys, dogs and sheep) (Rubin et al., 1988; Gross and Rubin, 1995; Li et al., 2005; Turner et al., 2006; Lloyd et al., 2012). Disuse-induced bone loss is associated with unbalanced bone remodeling (i.e. increased bone resorption and/or decreased bone formation), which leads to increased concentrations of serum and urinary calcium (Watanabe et al., 2004). Bone is also a calcium reservoir, containing 99% of the calcium in the body, and participates in systemic calcium homeostasis by helping maintain the minute-to-minute regulation of blood calcium concentration through bone remodeling (Green, 1994). Additionally, there are recently discovered roles for bone in the regulation of fat and energy metabolism (Karsenty and Ferron, 2012). Thus, along with mechanical loading, there are many competing physiological demands to which bone remodeling activity responds, ultimately influencing bone mass, strength and fracture risk (Doherty et al., 2015).

Hibernating bears are physically inactive for up to 6 months per year, but unlike most mammals, bears do not experience negative effects of this disuse period on bone structure and strength (McGee et al., 2008; McGee-Lawrence et al., 2009a,b). Thus, bears have evolved mechanisms to survive prolonged periods of extreme conditions without increased bone loss and fracture risk. This adaptation helps bears maintain the necessary physicality to forage and copulate (implantation is delayed until hibernation) following emergence from hibernation, promoting species survival. The biological mechanism that prevents bone loss in hibernating bears is unknown, but it was suggested that bone resorption and formation remain balanced in hibernating bears to help maintain calcium homeostasis (Donahue et al., 2006). It has been suggested bears do not excrete waste during hibernation (Nelson et al., 1984), although it is possible that they excrete miniscule amounts of waste during this period. It was previously proposed that bears recycle calcium released from bone by osteoclastic resorption back into bone via osteoblastic bone formation to help maintain homeostatic serum calcium concentration (Donahue et al., 2006), similar to the bear's ability to recycle urea nitrogen during hibernation (Nelson et al., 1984). Mechanistic data for these hypotheses, however, are conflicting. A cross-sectional static and dynamic histomorphometry study in hibernating and active grizzly bears, *Ursus arctos horribilis*, showed bone remodeling is decreased to 25% of summer levels during hibernation and bone resorption and formation activity are balanced (McGee et al., 2008), which would contribute to eucalcemia. In contrast, a cross-sectional study of serum markers of bone remodeling (bone formation: bone-specific alkaline phosphatase, BSALP; bone resorption: carboxy-terminal telopeptide of type 1 collagen, CTX) in black bears, *Ursus americanus*, indicated increased bone resorption with decreased bone formation during hibernation (Seger et al., 2011), whereas a longitudinal study of serum markers of bone remodeling (bone formation: C-terminal propeptide of type 1 procollagen, PICP;

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bone resorption: C-terminal cross-linked telopeptide of type 1 procollagen, ICTP) in black bears indicated that both resorption and formation markers increased during hibernation (Donahue et al., 2006). A possible explanation for the conflicting results from previous studies is that they analyzed circulating markers of bone remodeling that accumulate in serum with decreased renal function (Urena and de Vernejoul, 1999), and bears have decreased renal output during hibernation (Nelson et al., 1975). The bone formation marker BSALP and bone resorption marker TRACP (tartrate-resistant acid phosphatase) do not accumulate with advancing kidney dysfunction and decreased glomerular filtration rate (Fahrleitner-Pammer et al., 2008). Given decreased glomerular filtration rates in hibernating bears (Brown et al., 1971), BSALP and TRACP are among the most reliable bone remodeling markers but, to date, no studies on hibernating bears have measured these markers simultaneously in a longitudinal study.

Hibernation, in many species, is characterized by suppressed metabolism to conserve energy during prolonged fasting (Carey et al., 2003; Toien et al., 2011). For example, metabolic rate and heart rate are reduced to approximately 25% of summer levels in hibernating black bears (Toien et al., 2011); it is intriguing to note that intracortical bone remodeling in hibernating bears is also reduced to about 25% of summer levels (McGee et al., 2008). Because bone remodeling is metabolically expensive, increased bone remodeling, which typically occurs with disuse, during hibernation would compete for energy stores with other physiological functions critical for survival, such as cardiac output and respiration. It seems more likely that bone turnover would be reduced during hibernation, as other physiological functions are, to conserve energy, but results from previous studies have provided conflicting evidence. To better address mechanisms of bone turnover and calcium homeostasis during hibernation, seasonal changes in serum concentrations of calcium and bone remodeling markers that do not accumulate with decreased renal function (BSALP and TRACP) were quantified in hibernating black bears. Additionally, trans-ilial biopsies were obtained from two bears during pre-hibernation, hibernation and the transition out of hibernation to assess histological indices of bone remodeling longitudinally. Insulin, glucose, adiponectin and neuroendocrine factors also were quantified to assess energy metabolism as there is a switch from carbohydrate- to fat-based energy sources during hibernation (Carey et al., 2003) and there is a strong link between fat, energy and bone metabolism (Karsenty and Ferron, 2012).

RESULTS

Decreased serum blood urea nitrogen:creatinine ratios indicate hibernation status

The serum blood urea nitrogen (BUN):creatinine ratio showed significant (P=0.0004) seasonal variations. It was lowest in hibernating bears, confirming reduced renal clearance during hibernation (Table 1). Historically, a serum urea:creatinine ratio of less than 10, indicative of reduced renal function, has been considered the defining state of hibernation in bears that are on an adequate nutritional plane (Nelson et al., 1984). BUN levels were lowest and creatinine levels were highest in the hibernating bears, resulting in a BUN:creatinine ratio of 3.4 during hibernation compared with a ratio of 15.7 pre-hibernation. BUN:creatinine ratios of 6.1 and 10.5 for periods of transition into hibernation and out of hibernation, respectively, reflect that hibernation is not an all-or-none process, but rather is a gradual transition into and out of a hypometabolic state (Fig. 1). In the 10 bears for which body mass was measured at the beginning and end of hibernation, there was a 26.5±6.4% loss of body mass over the 3 month hibernation period.

Normal serum calcium concentration is maintained during hibernation

Neither total nor ionized serum calcium concentration showed significant (P>0.14) seasonal variation (Table 1, Fig. 2), confirming eucalcemia throughout the year as reported previously for total serum calcium (Floyd et al., 1990; Seger et al., 2011). Seven of the bears studied gave birth during hibernation to an average litter size of 2.1±0.4 cubs. Total serum calcium concentration was independent of cub birth, but ionized calcium concentration was significantly (P=0.033) lower (4%) in bears that gave birth.

Table 1. The concentrations of serum chemistry, energy metabolism, bone remodeling and endocrine parameters

	Pre-hibernation	Transition in	Hibernation	Transition out
Serum chemistry				
BUN:creatinine	15.7±5.5^{a}	6.1±1.2^{b,c}	3.4±2.0^{c}	10.5±4.3^{a,b}
Total calcium (mg dl^{-1})	8.7±0.4	8.7±0.2	8.6±0.2	8.3±0.3
Ionized calcium (mg dl^{-1})	3.7±0.1	3.7±0.2	3.6±0.2	3.8±0.1
Energy metabolism				
Glucose (mg dl^{-1})	165±49^{a,b}	185±34^{a}	120±16^{c}	139±41^{b,c}
Insulin (μIU ml^{-1})	8.5±3.2^{a}	6.4±2.0^{a,b}	5.7±1.7^{b}	7.5±3.4^{a,b}
Bone remodeling				
OCN (ng ml^{-1})	24.3±15.4^{a}	25.3±16.1^{a}	53.9±26.1^{b}	41.6±18.9^{b}
BSALP (U l^{-1})	20.0±16.3^{a}	13.4±9.3^{a,b}	9.6±3.2^{b}	21.2±10.7^{a}
TRACP (OD)	1.12±0.16^{a,b}	1.22±0.36^{a}	0.98±0.21^{b}	1.32±0.18^{a}
Endocrine				
Adiponectin (ng ml^{-1})	2013±434^{a}	1815±239^{a}	1333±177^{b}	1907±366^{a}
NPY (pmol l^{-1})	202±59^{a,b}	221±56^{a}	226±46^{a}	192±57^{b}
CART (ng ml^{-1})	8.0±10.8^{a}	35.7±36.7^{b}	119.2±52.6^{c}	16.7±10.9^{d}

Seasonal means±s.d. are presented; values with different superscript letters are significantly (P<0.05) different from one another. OD, optical density. Number of serum samples and bears used for each assay: BUN (blood urea nitrogen):creatinine, 63 samples from 5 bears; total calcium, 63 samples from 5 bears; ionized calcium, 95 samples from 7 bears; glucose, 167 samples from 10 bears; insulin, 170 samples from 10 bears; OCN (osteocalcin), 182 samples from 10 bears; BSALP (bone-specific alkaline phosphatase), 154 samples from 10 bears; TRACP (tartrate resistant acid phosphatase), 169 samples from 9 bears; adiponectin, 165 samples from 9 bears; NPY (neuropeptide Y), 186 samples from 5 bears; CART (cocaine and amphetamine regulated transcript), 97 samples from 5 bears.

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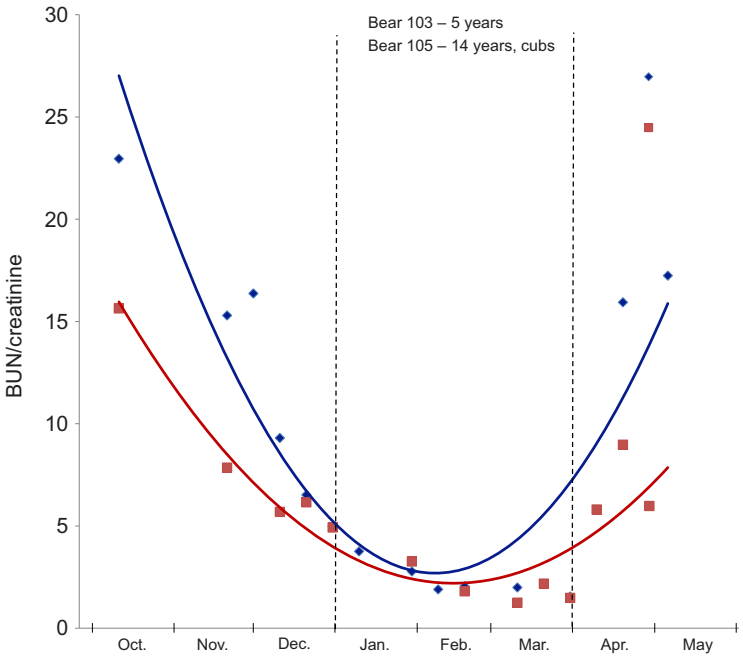

Fig. 1. The ratio of the serum concentrations of blood urea nitrogen (BUN) to creatinine in a representative sample of two black bears. One bear gave birth during hibernation (bear 105), the other did not (bear 103). Dashed lines represent the approximate start and end times of hibernation (January to March).

Bone remodeling is suppressed during hibernation

Serum concentrations of osteocalcin (OCN) were significantly (P=0.0001) elevated by 113% during hibernation relative to pre-hibernation levels (Table 1), similar to previous findings (Donahue et al., 2006). However, serum osteocalcin is known to accumulate and not reliably indicate bone formation during renal dysfunction (Fahrleitner-Pammer et al., 2008). Serum BSALP, which does not accumulate during decreased glomerular function, showed significant (P=0.007) seasonal variation; there was a gradual reduction from pre-hibernation to

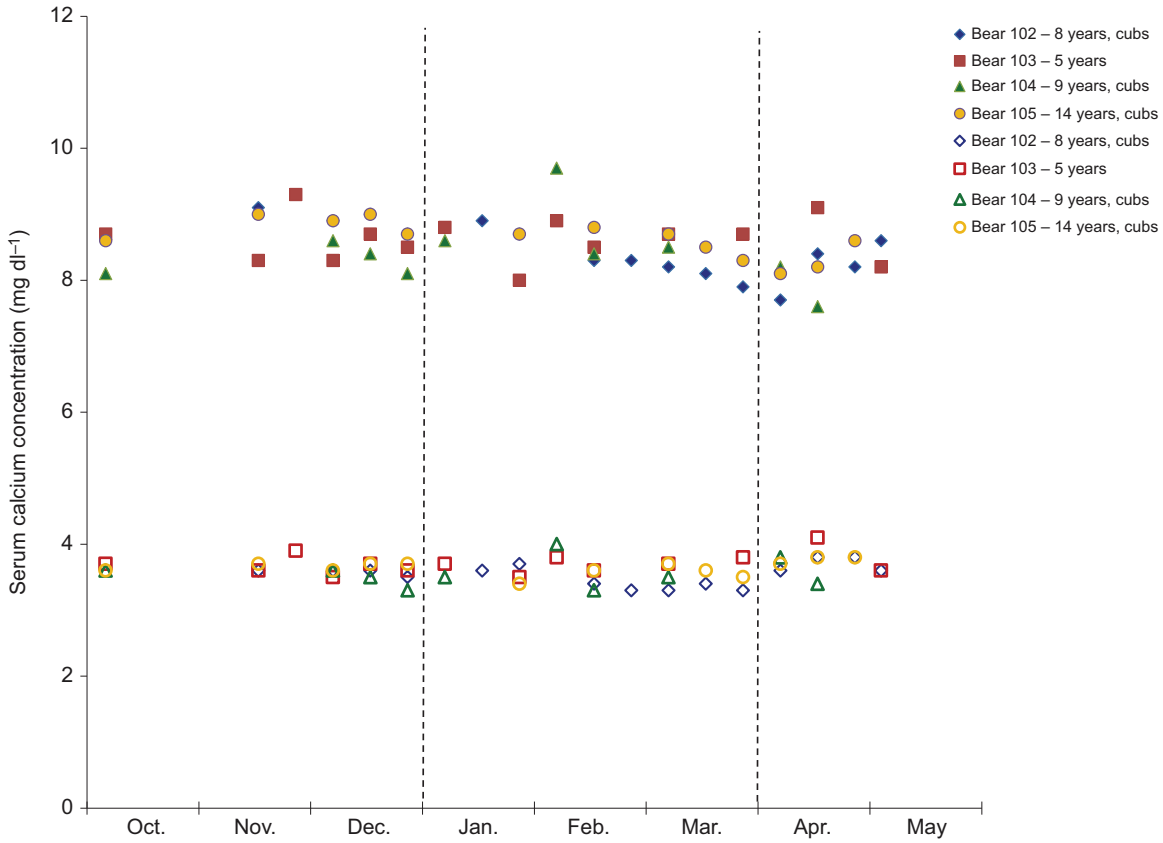

Fig. 2. Total and ionized calcium concentration in serum from four black bears. Filled symbols, total calcium concentration; open symbols, ionized calcium concentration. Dashed lines represent the approximate start and end times of hibernation (January to March). The age of the bear and the birth of cubs during the hibernation season are indicated in the key; only bear 103 did not have cubs during hibernation.

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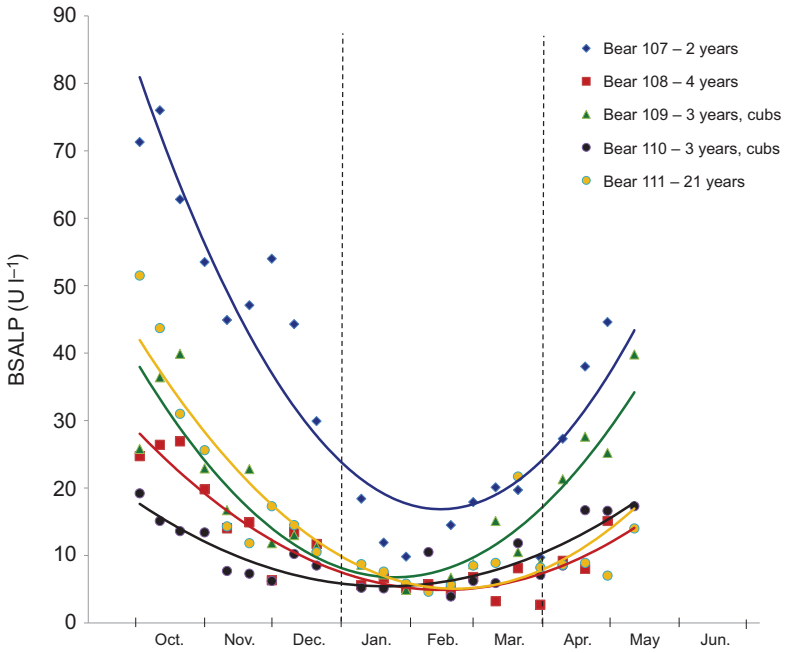

Fig. 3. The serum levels of the bone formation marker BSALP from five black bears. Dashed lines represent the approximate start and end times of hibernation (January to March). The age of the bear and the birth of cubs during the hibernation season are indicated in the key; only bear 109 and 110 had cubs during hibernation.

hibernation and an increase from hibernation through the transition out of hibernation, where it reached the pre-hibernation levels (Table 1, Fig. 3). Similarly, bone biopsies showed a reduced number of osteoblasts on bone surfaces during hibernation (0.15±0.11%) compared with pre-hibernation (1.96±0.29%) and the transition out of hibernation (9.37±12.23%) (Table 2). The serum level of TRACP also showed significant (P=0.003) changes, with a gradual decline from pre-hibernation to hibernation and a gradual increase during the transition out of hibernation (Fig. 4), reaching its lowest value during hibernation (Table 1), indicating reduced osteoclast number. Histological analyses also indicated reduced osteoclast number on bone surfaces during hibernation (0.63±0.78%) compared with pre-hibernation (1.45±0.46%) and the transition out of hibernation (4.01±2.46%) (Table 2). Serum TRACP levels were significantly (P=0.034) higher (20%) in sows that gave birth compared with non-parous females.

Serum cocaine and amphetamine regulated transcript concentration increases during hibernation

The serum concentration of cocaine and amphetamine regulated transcript (CART), a hormone known to reduce bone resorption (Singh et al., 2008), was significantly (P=0.002) different in all four seasons (Table 1). Lowest values were found in the pre-hibernation season and the concentration increased during the transition into hibernation and reached its highest level during hibernation. Following hibernation, CART concentration significantly decreased. The significant (P=0.004) seasonal changes in serum neuropeptide Y (NPY) concentration were relatively modest (Table 1). NPY is known to inhibit osteoblast and osteoclast cells (Shi and Baldock, 2012). The serum NPY concentration was lower during the transition out of hibernation compared with levels in hibernation and the transition into hibernation. Adiponectin, which can have negative effects on bone formation (Khor et al., 2013), was significantly (P=0.0006) lower during hibernation than in the other three time periods. Both serum glucose (P=0.0003) and insulin (P=0.012) were significantly lower during hibernation than in the pre-hibernation period (Table 1).

Table 2. Histological data from trans-ilial biopsies from two bears

Measured variable	Pre-hibernation	Hibernation	Transition out
ObS/BS (%)	1.96±0.29	0.15±0.11	9.37±12.23
OcS/BS (%)	1.45±0.46	0.63±0.78	4.01±2.46
MS/BS (%)	10.13±8.49	6.64±1.58	5.32±7.22
MAR (μm day^{-1})	1.70±0.52	1.67±0.57	1.59±0.44

Means±s.d. are presented.
ObS, osteoblast surface; OcS, osteoclast surface; MS, mineralizing surface; BS, bone surface; MAR, mineral apposition rate; Pre-hibernation, October; Hibernation, February; Transition out: April.

DISCUSSION

In most mammals, disuse leads to unbalanced bone remodeling and increases in serum calcium concentration and urinary calcium excretion (Weinreb et al., 1989; LeBlanc et al., 1995). This leads to weaker bones and increased fracture risk. Conversely, serum markers in the current study show hibernating black bears have reduced bone turnover during hibernation, and this is supported by histological studies (McGee et al., 2008; McGee-Lawrence et al., 2009b). Reduced bone remodeling during hibernation would contribute to the global metabolic suppression that occurs during hibernation (Toien et al., 2011) to conserve energy supplies (primarily fat) during the prolonged nutritional deprivation. The finding that serum calcium concentration is unchanged between hibernation and non-hibernation periods supports the idea that bone formation and resorption are balanced during hibernation, as histological studies suggest (McGee et al., 2008; McGee-Lawrence et al., 2009b), as bears are a closed system during hibernation (i.e. no calcium intake or excretion). Balanced bone resorption and formation activity maintains homeostatic serum calcium concentration, which is critical for maintaining extracellular fluid calcium concentrations and normal organ function (Green, 1994), and also preserves bone structure and strength (McGee et al., 2008; McGee-Lawrence et al., 2009a), which reduces fracture risk during remobilization upon emergence from hibernation. This unique ability to reduce bone turnover to conserve metabolic energy and prevent bone loss during prolonged periods of disuse contributes to bears' ability to survive prolonged periods of extreme environmental conditions.

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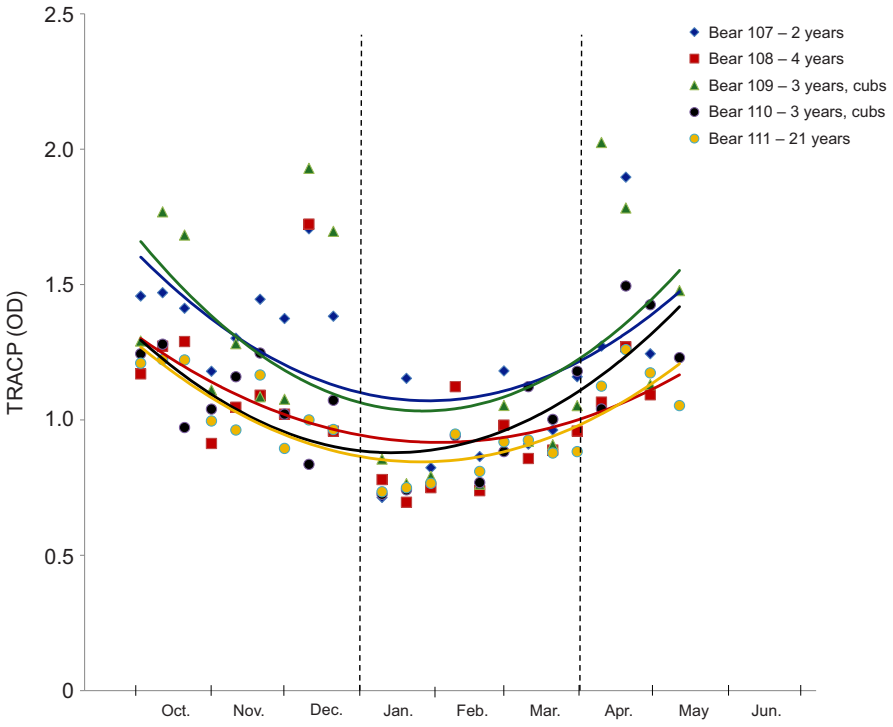

Fig. 4. The optical density (OD) for the serum marker of osteoclast number TRACP from five black bears. Dashed lines represent the approximate start and end times of hibernation (January to March). The age and the birth of cubs during the hibernation season are indicated for each bear in the key; only bear 109 and 110 had cubs during hibernation.

The serum level of the osteoclast marker TRACP was 20% higher in sows that gave birth as compared with those that did not. This raises the possibility that some calcium may be liberated from the maternal skeleton for milk production. The serum ionized calcium concentration was significantly (4%) lower in bears that gave birth, suggesting some calcium may be transferred from the sow to facilitate fetal development and lactation post-partum. Despite the possibility of increased bone resorption activity in sows that gave birth, female bears have bones that are mineralized to the same levels as male bears (McGee-Lawrence et al., 2009a). Bear cub birth mass is extremely low relative to maternal body mass: about 0.3% of maternal mass for bears compared with 1–5% for other carnivores (Oftedal et al., 1993). Thus, the extremely small cub birth mass may have evolved in part to protect the maternal skeleton as she does not ingest calcium during pregnancy and lactation. The small amount of calcium lost to cubs may be recovered when foraging resumes following hibernation.

Factors like adiponectin, CART and NPY, which are commonly associated with feeding and fat metabolism, can also have peripheral influences on bone metabolism. For example, *in vitro* and clinical studies suggest adiponectin has negative effects on bone formation (Khor et al., 2013). Thus, reduced circulating adiponectin during hibernation possibly removes an inhibitory effect on osteoblasts, allowing bone formation to maintain balance with bone resorption even though osteoblasts are challenged by reduced mechanical loading during the physical inactivity that occurs during hibernation. Low adiponectin is also linked to insulin resistance (Caselli, 2014). Thus, reduced adiponectin during hibernation also likely promotes the insulin resistance and lypolytic state observed in hibernating bears (Nelson et al., 2014). Peripheral NPY inhibits the number and activity of both osteoblast and osteoclast cells (Shi and Baldock, 2012). Thus, the high circulating NPY levels in hibernating bears may contribute to the decreased bone remodeling activity during hibernation. Central CART and NPY are well known for their effects on the regulation of feeding. However, the endocrine actions of peripheral CART, produced primarily in the pituitary and pancreas, may be more important in the regulation of bone remodeling than central CART (Singh et al., 2008). Overexpression of peripheral CART was shown to inhibit osteoclastogenesis and rescue the low bone mass phenotype of CART knockout mice. Serum CART was approximately 15 times higher in hibernating bears than in pre-hibernation bears, raising the possibility that it has a potent inhibitory effect on bone resorption during hibernation. This hypothesis is consistent with findings from the current study of lower values of serum TRACP and histological indices of osteoclast number during hibernation, and the previous finding of reduced activation frequency of bone remodeling units in hibernating grizzly bears (McGee et al., 2008). However, at present, it is unclear whether these results are correlative, or whether CART may act as an effector molecule in mechanisms regulating bone remodeling in hibernating bears. Ongoing and future studies, including proteomics analyses, will be necessary to better understand the role of peripheral CART in hibernating bear physiology.

Fat is the primary energy source bears use to survive hibernation (Hellgren, 1998). Black bears lose approximately 25% of their body mass, almost exclusively fat mass, during hibernation (Hellgren et al., 1990). Hyperphagia prior to hibernation promotes the accumulation of large fat stores and there is a transition from carbohydrate oxidation to fat combustion during hibernation (Carey et al., 2003). Consistent with this, we found that serum levels of glucose significantly decreased between pre-hibernation and hibernation seasons (Table 1). Insulin also decreased from pre-hibernation to hibernation periods, similar to findings in grizzly bears, which show insulin resistance without hyperglycemia during hibernation (Nelson et al., 2014). Insulin signaling in osteoblasts triggers decreased production of osteoprotegerin and subsequent activation of osteoclast-mediated bone resorption (Karsenty and Ferron, 2012). Thus, reduced circulating insulin during hibernation may help promote reduced bone remodeling, which would contribute to energy conservation. In mice, insulin signaling in osteoblasts is associated with undercarboxylated osteocalcin-induced insulin secretion from beta cells (Karsenty and Ferron, 2012). Osteocalcin

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also promotes adiponectin expression in the adipocytes of mice (Lee and Karsenty, 2008). We were unable to measure undercarboxylated osteocalcin in bear serum, but it is strongly correlated with total osteocalcin in other species and total osteocalcin is inversely correlated with glucose metabolism in humans (Booth et al., 2013). In bears, elevated serum osteocalcin during hibernation coincided with decreased insulin and adiponectin concentrations; however, the increase in the serum concentration of total osteocalcin during hibernation is likely a result of reduced renal clearance (Fahrleitner-Pammer et al., 2008). It is also possible that increased concentrations of osteocalcin fragments contributed to the increased osteocalcin concentration found during hibernation (Calvo et al., 1996). Thus, it is difficult to interpret the role osteocalcin plays in energy metabolism in hibernating bears.

It should be noted that serum markers of bone metabolism reflect net bone metabolism from the entire skeleton and that it is possible that focal regions within the skeleton show different seasonal changes in bone remodeling from those indicated by serum markers. It is conceivable that repeated anesthesia during the study period influenced the serum measurements. However, the anesthesia administration was consistent throughout the study and none of the outcome variables increased or decreased continuously over the entire study period (i.e. with repeated anesthesia). In contrast, they showed reversible changes (e.g. BSALP) or no changes (e.g. calcium) over the duration of the study period. It is also possible that dietary differences between wild and captive conditions influence bone metabolism. However, all the samples that were analyzed in this study were collected during captive conditions, and thus the relative seasonal comparisons are from bears that were on the same diet (high protein dog food).

A myriad of neural, endocrine, paracrine and mechanical signals influence bone mass and strength during development, senescence and various disease states. The analysis of the hormones that can influence bone remodeling in this study is by no means complete, but results from the current work do provide novel insight into the possible roles of insulin, adiponectin, NPY and CART in regulating the unique bone remodeling processes that bears have evolved to avoid the deleterious consequences of prolonged disuse and to survive, and reproduce during, the extreme environmental conditions of hibernation. Additionally, the longitudinal analyses of serum calcium and bone remodeling markers (i.e. BSALP and TRACP) that do not accumulate with decreased renal function support the idea that bone remodeling is decreased, but balanced, during hibernation. Hibernating bears are metabolic marvels (Nelson, 1987) that have the ability to be physically inactive and obese without experiencing complications such as heart disease, skeletal muscle atrophy and diabetes (Harlow et al., 2001; Nelson et al., 2003, 2014). Integrative physiology and systems biology approaches will undoubtedly shed new light on the mechanisms of the unique metabolism of numerous tissues and organs in hibernating bears and have transformative impact on novel approaches to understanding and treating human diseases.

MATERIALS AND METHODS

Bear handling and blood sample collection

The Virginia Polytechnic Institute and State University Animal Care Committee approved all bear handling protocols (no. 98-069-F&WS). A total of 13 female black bears between 2006 and 2009 were captured and held in pens in the Virginia Tech Center for Bear Research from autumn through to spring, representing one full hibernation season. Upon completion of the study, the bears were released. The bears were between 2 and 21 years old, and many gave birth to cubs during hibernation; information regarding ages and cub births are provided in Table 3. Bears demonstrating behavior indicative of stress, such as pacing, were released and not used in the study. Bears were fed 2000 g of high protein (25–27% protein) dog food daily from the date of capture until 1 December. Beginning on 1 December, the amount of provided food was reduced by 50% every 10 days until feeding was stopped completely on December 31. Food was reintroduced to the bears on the first of April upon emergence from hibernation.

Table 3. Age, number of offspring and year of blood collection for the bears used in this study

Bear ID	Age	No. of cubs	Year
98	Unknown	0	2006–7
99	Unknown	2	2006–7
100	2	0	2007–8
101	4	2	2007–8
102	8	2	2007–8
103	5	0	2007–8
104	9	2	2007–8
105	14	2	2007–8
107	2	0	2008–9
108	4	0	2008–9
109	3	2	2008–9
110*	3	3	2008–9
111*	21	0	2008–9

Asterisks indicate bears from which biopsies were obtained for histological analyses.

To facilitate blood sample collection during the study, bears were anesthetized via pneumatic dart with a 2:1 mixture of ketamine (100 mg ml^{-1}):xylazine (100 mg ml^{-1}); the dosage was 1 ml of the mixture per 45.5 kg of body mass. Blood samples were drawn from the femoral vein while the bears were anesthetized, and the samples were transported to the laboratory in an ice-packed cooler. Immediately upon arrival in the laboratory, the blood was spun to isolate the serum, which was partitioned into aliquots and frozen at −80°C. Blood samples were collected from each bear every 10 days from the beginning of October until the end of May. Hibernation began in early January and ended in early April. Thus, the collection dates encompassed an active pre-hibernation period, a disuse hibernation period and a post-hibernation remobilization period, including the transitory periods between seasons (defined below).

To assess the effects of hibernation on bone, calcium and energy metabolism, serum concentrations of BUN, creatinine, total and ionized calcium, bone remodeling markers, glucose, insulin, adiponectin, NPY and CART were quantified. Bone remodeling markers that do not accumulate with renal dysfunction were selected for analysis. TRACP is an indicator of osteoclast number and BSALP is an indicator of osteoblastic bone formation. The serum marker of bone formation osteocalcin, which does accumulate with renal dysfunction, was also quantified for comparison with previous findings (Donahue et al., 2006). Additionally, trans-ilial biopsies (8 mm diameter) were obtained from two bears during pre-hibernation, hibernation and the transition out of hibernation time points to assess histological indices of bone remodeling.

The entrance into and emergence from hibernation are not immediate processes. Instead, metabolism is gradually suppressed over a period of weeks while the bears are still active outside of the hibernacula, during which time the animals experience reduced physical activity and feeding. Similarly, after emergence from the hibernacula, activity, feeding and metabolism are gradually increased. Black bears require approximately 2–3 weeks following emergence to reach summer basal metabolic rate (BMR) (Toien et al., 2011). While in the den, black bear metabolism remains steadily suppressed to approximately 25% of summer BMR (Toien et al., 2011). This physiological pattern is reflected by renal activity, measured as serum ratios between urea and creatinine; hibernation BUN:creatinine ratios are less than half of summer values (Nelson et al., 1984). There is a gradual decrease in the BUN:creatinine ratio, prior to the onset of hibernation, that corresponds approximately to the 30 day period of reduced food intake (Hellgren et al., 1990). Based on these findings, four periods corresponding to metabolic status were defined for the collected serum samples:

pre-hibernation (10 October to 30 November), transition into hibernation (10–30 December), hibernation (10 January to 30 March) and transition out of hibernation (10 April to 10 May).

Fluorochrome labeling and iliac biopsy

Trans-ilial biopsies (8 mm diameter) were available from only two bears during pre-hibernation, hibernation and post-hibernation time points as previously described (Floyd et al., 1990); biopsy dates were 20 October 2008, 28 February 2009 and 30 April 2009, respectively. The right ilium was used for pre- and post-hibernation biopsies and the left ilium was used for biopsies during hibernation. The second set of biopsies from the right ilium were taken approximately 5 cm away from the first biopsy site to avoid sampling the previous site and any subsequent artifact from ongoing wound-healing processes (Rao, 1983). Bears were administered i.v. solutions of tetracycline (15 mg kg^{-1} body mass; October), calcein (5 mg kg^{-1} body mass; February) or alizarin complexone (20 mg kg^{-1} body mass; April) twice prior to each biopsy procedure; injections were given 10 days apart, and 9–11 days passed after the second label was administered before samples were harvested. Biopsies were fixed in 10% neutral buffered formalin and stored in 70% ethanol prior to being histologically processed as previously described (McGee-Lawrence et al., 2009b). Osteoblast surface (osteoblast surface/bone surface, %), osteoclast surface (osteoclast surface/bone surface, %), mineralizing surface (mineralizing surface/bone surface, %) and mineral apposition rate (μm day^{-1}) were calculated for each biopsy.

Biochemical assays: bone remodeling

Serum TRACP activity was quantified as follows: 10 μl of plasma from the bears was diluted in 10 μl MilliQ water, and added to 80 μl of freshly prepared reaction buffer (0.33 mol l^{-1} acetic acid, 0.167% Triton X-100, 0.33 mol l^{-1} NaCl, 3.33 mmol l^{-1} EDTA at pH 5.5, 1.5 mg ml^{-1} ascorbic acid, 7.66 mg ml^{-1} Na_2-tartrate, 3 mg ml^{-1} 4-nitrophenylphosphate). The optical density at 405 nm, with reference 650 nm, was quantified. Relative optical densities are reported as no reference enzyme for TRACP exists. Notwithstanding, there is good correlation between serum optical density values for TRACP and osteoclast numbers in bone (Henriksen et al., 2007).

Activities of BSALP and total alkaline phosphatase (ALP) were determined by differential binding to wheat germ lectin (WGL) as follows: 25 μl of serum was mixed with 25 μl of WGL solution (4.5 mg ml^{-1}) and the mixture was incubated at 37°C for 30 min. The mixture was centrifuged for 2 min, and alkaline phosphatase activities of the supernatant and parallel untreated serum were quantified by a kinetic colorimetric assay at an optical density of 405 nm (Sigma, St Louis, MO, USA). Total serum ALP was the activity of the untreated serum. BSALP activity was quantified by subtracting the WGL supernatant from the total ALP activity. Serum OCN was quantified as previously described (Donahue et al., 2006). Briefly, 10 μl bear serum was assayed in duplicate by radioimmunoassay (RIA). The antibody was guinea-pig anti-rat OCN and the tracer was ^{125}I-labeled rat OCN. Dose dilutions of purified bear OCN (Donahue et al., 2006) were used as standards. Intra-sample measurements varied by less than 5%.

Biochemical assays: serum chemistry and metabolism

Insulin was quantified using the Millipore (Linco Research Inc., St Louis, MO, USA) RIA method. Sensitivity of the method is 2 IU ml^{-1}. Intra-assay precision averaged 3.2% while inter-assay precision averaged 3.9%. Chemistry panels (including total calcium, glucose, BUN and creatinine) were performed on a Roche modular P Clinical Chemistry analyzer (Roche, Nutley, NJ, USA). Kidney function was assessed by calculating BUN: creatinine ratios. Ionized calcium was quantified with an ion-selective electrode (Bayer Rapidlab 865, Leverkusen, Germany). Adiponectin was measured by the human adiponectin double antibody RIA kit (Linco Research Inc.).

Biochemical assays: neuroendocrine peptides

NPY was quantified using a competitive RIA method with reagents manufactured by EURIA and distributed by ALPCO (Salem, NH, USA). The sensitivity of the NPY RIA method was 3 pmol l^{-1}; intra-assay precision averaged 4.8% while inter-assay precision averaged 8.4%. Serum CART levels were quantified with a commercial enzyme immunoassay (EIA; RayBio no. EIA-CART-1). The sensitivity of the CART assay was 13.5 pg ml^{-1}.

Statistics

Because of limitations in the volume of serum obtained at each sampling time point, it was not possible to analyze samples from every time point for each bear for each assay. The number of serum samples and number of bears used for each assay are given in the legend to Table 1. Seasonal changes are shown graphically for bears that had sufficient data points in all seasons. To better facilitate seasonal comparisons, longitudinal data points from each bear were grouped and categorized via seasonal hibernation status. Bears experience a transitional state beginning roughly 30 days prior to the onset of hibernation and ending approximately 30 days after emergence from denning where metabolic processes remain suppressed despite the presence of physical activity (Nelson et al., 1983, 1984). To account for this phenomenon, serum samples collected up to 1 December of each year were designated as 'pre-hibernation', whereas samples collected between 1 and 31 December of each year (during which time food access was systematically reduced) were designated as 'transition into hibernation'. Samples collected between 1 January and 31 March of each year were designated as hibernation, and any samples collected after April 1, when feeding was re-introduced, were categorized as 'transition out of hibernation'. For each serum assay, the mean value for each bear within each season was calculated and used for analysis of seasonal changes. Data were analyzed with 2-factor ANOVA (factor 1=season, factor 2=bear) and seasonal comparisons were subsequently performed using Tukey's highly significant difference (HSD) *post hoc* test.

All of the bears used for the study were female, and many gave birth to cubs over the course of the hibernation season. To determine whether parity and lactation affected seasonal trends in serum markers, data were analyzed with 2-factor ANOVA with interaction (factor 1=presence of cubs, 'yes' or 'no', factor 2=season, interaction=parity×season). A significance of $P<0.05$ was used for all analyses. Because of the small sample size for the histological data ($N=2$ per season), statistical comparisons were not performed; data are presented as means±s.d. for qualitative purposes only.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.M-L. analyzed the data and prepared the manuscript, P.B. developed and executed the osteocalcin procedures and revised the manuscript, C.C. developed and executed the BSALP procedures and revised the manuscript, K.H. developed and executed the TRACP procedures and revised the manuscript, M.V. oversaw animal handling and sampling procedures and revised the manuscript, S.D. developed the concepts and approach and prepared the manuscript.

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References

Booth, S. L., Centi, A., Smith, S. R. and Gundberg, C. (2013). The role of osteocalcin in human glucose metabolism: marker or mediator? *Nat. Rev. Endocrinol.* **9**, 43-55.

Brown, D. C., Mulhausen, R. O., Andrew, D. J. and Seal, U. S. (1971). Renal function in anesthetized dormant and active bears. *Am. J. Physiol.* **220**, 293-298.

Calvo, M. S., Eyre, D. R. and Gundberg, C. M. (1996). Molecular basis and clinical application of biological markers of bone turnover. *Endocr. Rev.* **17**, 333-368.

Carey, H. V., Andrews, M. T. and Martin, S. L. (2003). Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. *Physiol. Rev.* **83**, 1153-1181.

Caselli, C. (2014). Role of adiponectin system in insulin resistance. *Mol. Genet. Metab.* **113**, 155-160.

Doherty, A. H., Ghalambor, C. K. and Donahue, S. W. (2015). Evolutionary physiology of bone: bone metabolism in changing environments. *Physiology* **30**, 17-29.

Donahue, S. W., Galley, S. A., Vaughan, M. R., Patterson-Buckendahl, P., Demers, L. M., Vance, J. L. and McGee, M. E. (2006). Parathyroid hormone may maintain bone formation in hibernating black bears (*Ursus americanus*) to prevent disuse osteoporosis. *J. Exp. Biol.* **209**, 1630-1638.

Fahrleitner-Pammer, A., Herberth, J., Browning, S. R., Obermayer-Pietsch, B., Wirnsberger, G., Holzer, H., Dobnig, H. and Malluche, H. H. (2008). Bone markers predict cardiovascular events in chronic kidney disease. *J. Bone Miner. Res.* **23**, 1850-1858.

Floyd, T., Nelson, R. A. and Wynne, G. F. (1990). Calcium and bone metabolic homeostasis in active and denning black bears (*Ursus americanus*). *Clin. Orthop.* **255**, 301-309.

Green, J. (1994). The physicochemical structure of bone: cellular and noncellular elements. *Miner. Electrolyte Metab.* **20**, 7-15.

Gross, T. S. and Rubin, C. T. (1995). Uniformity of resorptive bone loss induced by disuse. *J. Orthop. Res.* **13**, 708-714.

Harlow, H. J., Lohuis, T., Beck, T. D. I. and Iaizzo, P. A. (2001). Muscle strength in overwintering bears. *Nature* **409**, 997.

Hellgren, E. C. (1998). Physiology of hibernation in bears. *Ursus* **10**, 467-477.

Hellgren, E. C., Vaughan, M. R., Kirkpatrick, R. L. and Scanlon, P. F. (1990). Serial changes in metabolic correlates of hibernation in female black bears. *J. Mammal.* **71**, 291-300.

Henriksen, K., Tanko, L. B., Qvist, P., Delmas, P. D., Christiansen, C. and Karsdal, M. A. (2007). Assessment of osteoclast number and function: application in the development of new and improved treatment modalities for bone diseases. *Osteoporos. Int.* **18**, 681-685.

Karsenty, G. and Ferron, M. (2012). The contribution of bone to whole-organism physiology. *Nature* **481**, 314-320.

Khor, E. C., Wee, N. K. Y. and Baldock, P. A. (2013). Influence of hormonal appetite and energy regulators on bone. *Curr. Osteoporos. Rep.* **11**, 194-202.

LeBlanc, A., Schneider, V., Spector, E., Evans, H., Rowe, R., Lane, H., Demers, L. and Lipton, A. (1995). Calcium absorption, endogenous excretion, and endocrine changes during and after long-term bed rest. *Bone* **16**, 301S-304S.

Lee, N. K. and Karsenty, G. (2008). Reciprocal regulation of bone and energy metabolism. *Trends Endocrinol. Metab.* **19**, 161-166.

Li, C. Y., Price, C., Delisser, K., Nasser, P., Laudier, D., Clement, M., Jepsen, K. J. and Schaffler, M. B. (2005). Long-term disuse osteoporosis seems less sensitive to bisphosphonate treatment than other osteoporosis. *J. Bone Miner. Res.* **20**, 117-124.

Lloyd, S. A., Bandstra, E. R., Willey, J. S., Riffle, S. E., Tirado-Lee, L., Nelson, G. A., Pecaut, M. J. and Bateman, T. A. (2012). Effect of proton irradiation followed by hindlimb unloading on bone in mature mice: a model of long-duration spaceflight. *Bone* **51**, 756-764.

McGee, M. E., Maki, A. J., Johnson, S. E., Nelson, O. L., Robbins, C. T. and Donahue, S. W. (2008). Decreased bone turnover with balanced resorption and formation prevent cortical bone loss during disuse (hibernation) in grizzly bears (*Ursus arctos horribilis*). *Bone* **42**, 396-404.

McGee-Lawrence, M. E., Wojda, S. J., Barlow, L. N., Drummer, T. D., Bunnell, K., Auger, J., Black, H. L. and Donahue, S. W. (2009a). Six months of disuse during hibernation does not increase intracortical porosity or decrease cortical bone geometry, strength, or mineralization in black bear (*Ursus americanus*) femurs. *J. Biomech.* **42**, 1378-1383.

McGee-Lawrence, M. E., Wojda, S. J., Barlow, L. N., Drummer, T. D., Castillo, A. B., Kennedy, O., Condon, K. W., Auger, J., Black, H. L., Nelson, O. L. et al. (2009b). Grizzly bears (*Ursus arctos horribilis*) and black bears (*Ursus americanus*) prevent trabecular bone loss during disuse (hibernation). *Bone* **45**, 1186-1191.

Nelson, R. A. (1987). Black bears and polar bears—still metabolic marvels. *Mayo Clin. Proc.* **62**, 850-853.

Nelson, R. A., Jones, J. D., Wahner, H. W., McGill, D. B. and Code, C. F. (1975). Nitrogen metabolism in bears: urea metabolism in summer starvation and in winter sleep and role of urinary bladder in water and nitrogen conservation. *Mayo Clin. Proc.* **50**, 141-146.

Nelson, R. A., Streiger, D. L. and Beck, T. D. (1983). Neuroendocrine and metabolic interactions in the hibernating black bear. *Acta Zool. Fennica.* **174**, 137-141.

Nelson, R. A., Beck, T. D. and Steiger, D. L. (1984). Ratio of serum urea to serum creatinine in wild black bears. *Science* **226**, 841-842.

Nelson, O. L., McEwen, M.-M., Robbins, C. T., Felicetti, L. and Christensen, W. F. (2003). Evaluation of cardiac function in active and hibernating grizzly bears. *J. Am. Vet. Med. Assoc.* **223**, 1170-1175.

Nelson, O. L., Jansen, H. T., Galbreath, E., Morgenstern, K., Gehring, J. L., Rigano, K. S., Lee, J., Gong, J., Shaywitz, A. J., Vella, C. A. et al. (2014). Grizzly bears exhibit augmented insulin sensitivity while obese prior to a reversible insulin resistance during hibernation. *Cell Metab.* **20**, 376-382.

Oftedal, O. T., Alt, G. L., Widdowson, E. M. and Jakubasz, M. R. (1993). Nutrition and growth of suckling black bears (*Ursus americanus*) during their mothers' winter fast. *Br. J. Nutr.* **70**, 59-79.

Rao, D. S. (1983). Practical approach to bone biopsy. In Bone Histomorphometry: Techniques and Interpretation (ed. R. R. Recker), pp. 3-11. Boca Raton, FL: CRC Press.

Rubin, C. T., Pratt, G. W., Jr, Porter, A. L., Lanyon, L. E. and Poss, R. (1988). Ultrasonic measurement of immobilization-induced osteopenia: an experimental study in sheep. **42**, 309-312.

Seger, R. L., Cross, R. A., Rosen, C. J., Causey, R. C., Gundberg, C. M., Carpenter, T. O., Chen, T. C., Halteman, W. A., Holick, M. F., Jakubas, W. J. et al. (2011). Investigating the mechanism for maintaining eucalcemia despite immobility and anuria in the hibernating American black bear (*Ursus americanus*). *Bone* **49**, 1205-1212.

Shi, Y.-C. and Baldock, P. A. (2012). Central and peripheral mechanisms of the NPY system in the regulation of bone and adipose tissue. *Bone* **50**, 430-436.

Singh, M. K., Elefteriou, F. and Karsenty, G. (2008). Cocaine and amphetamine-regulated transcript may regulate bone remodeling as a circulating molecule. *Endocrinology* **149**, 3933-3941.

Toien, O., Blake, J., Edgar, D. M., Grahn, D. A., Heller, H. C. and Barnes, B. M. (2011). Hibernation in black bears: independence of metabolic suppression from body temperature. *Science* **331**, 906-909.

Turner, R. T., Lotinun, S., Hefferan, T. E. and Morey-Holton, E. (2006). Disuse in adult male rats attenuates the bone anabolic response to a therapeutic dose of parathyroid hormone. *J. Appl. Physiol.* **101**, 881-886.

Urena, P. and de Vernejoul, M.-C. (1999). Circulating biochemical markers of bone remodeling in uremic patients. *Kidney Int.* **55**, 2141-2156.

Watanabe, Y., Ohshima, H., Mizuno, K., Sekiguchi, C., Fukunaga, M., Kohri, K., Rittweger, J., Felsenberg, D., Matsumoto, T. and Nakamura, T. (2004). Intravenous pamidronate prevents femoral bone loss and renal stone formation during 90-day bed rest. *J. Bone Miner. Res.* **19**, 1771-1778.

Weinreb, M., Rodan, G. A. and Thompson, D. D. (1989). Osteopenia in the immobilized rat hind limb is associated with increased bone resorption and decreased bone formation. *Bone* **10**, 187-194.