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Bone formation is not impaired by hibernation (disuse) in black bears *Ursus americanus*

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Summary

Disuse by bed rest, limb immobilization or space flight causes rapid bone loss by arresting bone formation and accelerating bone resorption. This net bone loss increases the risk of fracture upon remobilization. Bone loss also occurs in hibernating ground squirrels, golden hamsters, and little brown bats by arresting bone formation and accelerating bone resorption. There is some histological evidence to suggest that black bears *Ursus americanus* do not lose bone mass during hibernation (i.e. disuse). There is also evidence suggesting that muscle mass and strength are preserved in black bears during hibernation. The question of whether bears can prevent bone loss during hibernation has not been conclusively answered. The goal of the current study was to further assess bone metabolism in hibernating black bears. Using the same serum markers of bone remodeling used to evaluate human patients with osteoporosis, we assayed serum from five black bears, collected every 10 days over a 196-day period, for bone resorption and formation markers. Here we show that bone resorption remains elevated over the entire hibernation period compared to the pre-hibernation period, but osteoblastic bone formation is not impaired by hibernation and is rapidly accelerated during remobilization following hibernation.

Key words: black bear, *Ursus americanus*, bone formation, hibernation, metabolism, adaptation, collagen, disuse.

Introduction

Disuse osteoporosis occurs in patients with spinal cord injuries, patients confined to prolonged bed rest, and astronauts exposed to microgravity during space flight (Collet et al., 1997; Dauty et al., 2000; Garland et al., 1992; Leblanc et al., 1990; Vico et al., 2000). Disuse osteoporosis also occurs in areas of low bone stress around orthopaedic implants and from limb immobilization after surgery (Houde et al., 1995; Lewis et al., 1998; Marchetti et al., 1996). During remobilization, the recovery of the bone lost during disuse is slow and may not be completely recoverable (Jaworski and Uhthoff, 1986; Leblanc et al., 1990; Lindgren and Mattsson, 1977; Vico et al., 2000). In hibernating ground squirrels, golden hamsters and little brown bats, bone is lost by reduced osteoblastic formation and increased bone resorption (Haller and Zimny, 1977; Kwiecinski et al., 1987; Steinberg et al., 1979, 1981, 1986). Other animal studies have shown that immediate rapid increases in bone resorption and sustained decreases in bone formation contribute to bone loss during limb immobilization by casting, tenotomy or neurectomy (Rantakokko et al., 1999; Weinreb et al., 1989). Following 2 weeks of hind limb immobilization and 4 weeks of remobilization, the bending strength, apparent density and degree of mineralization of rat femurs were significantly lower than in age-matched controls (Trebacz, 2001). In other studies where remobilization did restore the bone lost by immobilization, the recovery period was 2–3 times longer than the immobilization period (Kaneps et al., 1997; Weinreb et al., 1997). We recently found that porosity, mineral content and ultimate bending stress did not change with age in black bears *Ursus americanus*, despite annual periods of disuse (Harvey and Donahue, 2003). Since wild black bears hibernate for 5–7 months annually, the findings of these previous studies raise an important question regarding the regulation of bone mass and mechanical strength in hibernating black bears: how can black bears maintain bone mass and strength when annual hibernation (i.e. disuse) and active periods are approximately equal (i.e. 6 months)?

Approximately 90% of the organic component of the extracellular matrix of bone is type I collagen. The serum concentrations of type I collagen peptide fragments are useful for assessing bone turnover in patients with osteoporosis (Watts, 1999). The bone-forming osteoblasts secrete type I procollagen molecules, which have a central triple helical domain and telopeptide and propeptide domains on both the amino- and carboxy-terminal ends of the molecule. During

bone formation, the carboxy-terminal propeptide of type I procollagen (PICP) is cleaved off and released into the circulation. The concentration of PICP in serum has been positively correlated with histomorphometric measurements of bone formation (Eriksen et al., 1993). During resorption of bone, the cross-linked telopeptides are released into the serum. The degradation of type I bone collagen by the proteinase cathepsin K produces cross-linked amino-terminal telopeptide (NTX) fragments (Atley et al., 2000). The degradation of type I bone collagen by matrix metalloproteinases produces cross-linked carboxy-terminal telopeptide (ICTP) fragments (Garnero et al., 2003). However, cathepsin K destroys the ICTP epitope. Thus, different modes of enzymatic digestion of type I bone collagen produce different telopeptide fragments. The serum ICTP concentration has been positively correlated with bone resorption and negatively correlated with bone mineral density (Eriksen et al., 1993; Yasumizu et al., 1998). In patients immobilized for 30 to 180 days following a stroke, PICP levels were significantly lower and ICTP levels significantly higher than in healthy age-matched male and female controls (Fiore et al., 1999). Serum NTX levels are significantly (approximately twofold) higher in post-menopausal women than in pre-menopausal women (Garnero et al., 1996). These finding suggest that there are different mechanisms of collagen degradation for disuse and post-menopausal osteoporosis.

Hibernating black bears have been called ‘metabolic marvels’ for their unique physiological characteristics (Nelson, 1987). For example, hibernating bears remain dormant for 5–7 months, during which time they do not urinate or defecate, they efficiently recycle urea and amino acids, and the females give birth and nurse (Nelson, 1987; Wright et al., 1999). There is some evidence to suggest that bears do not lose bone or muscle mass during hibernation (Floyd et al., 1990; Tinker et al., 1998). Histomorphometric analyses of bone biopsies from bears showed that bone resorption and formation surfaces increased several-fold during winter hibernation relative to active summer values, and bone volume was unchanged (Floyd et al., 1990). Floyd et al. proposed that bears produce an osteoregulatory substance, which promotes increased bone formation during disuse to compensate for increased resorption, thus making bears uniquely resistant to disuse osteoporosis (Floyd et al., 1990).

Hibernating black bears provide a unique and naturally occurring model for studying the physiology of bone disuse with inactivity. Recently, we assayed serum PICP and ICTP levels from 17 wild black bears, collected during active and hibernating periods, using radioimmunoassay (Donahue et al., 2003). As in human bed-rest studies (Fiore et al., 1999), we found that serum ICTP concentration significantly increased in bears during hibernation (i.e. disuse). However, unlike human disuse studies, PICP levels were unchanged during hibernation in black bears when compared to the active period prior to hibernation. These findings suggested that bone resorption and formation was unbalanced during hibernation, resulting in net bone loss. However, in the months immediately following their arousal from hibernation there was a 3–4-fold increase in PICP levels in both a young and an old (17 year) bear, suggesting accelerated bone formation during remobilization, which was not compromised with aging. These findings on bear bone metabolism were provocative; however, in that study only one sample was collected from each bear during each period (immobilization and remobilization). Therefore, the time course of seasonal variations in serum markers of bone resorption and formation was not defined in our previous study. The goals of the current study were (1) to assess the time-course of seasonal variations in bone resorption and formation markers to find out if the changes in bone resorption and formation are sustained during the entire disuse period, which would give an indication of the amount of bone lost, and (2) to gain some insight on how the lost bone may be recovered during remobilization.

Materials and methods

Blood samples were collected from five black bears *Ursus americanus* Pallas held in a captive bear facility. The Virginia Polytechnic Institute and State University Animal Care Committee approved all bear handling protocols (#98-069-F&WS). The bears were anesthetized with a 2:1 mixture of ketamine (100 mg ml^{-1}):xylazine (100 mg ml^{-1}); the dosage was 1 cc of the mixture per 45 kg of body mass. Body temperatures were 4–6°C cooler during winter collection, confirming that the bears were in a state of hibernation. No urine or scat was present in the hibernation dens. Stressful behavior was not observed during any of the handling procedures. 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 on return to the laboratory, the blood was spun to isolate the serum, which was frozen at –28°C to –51°C. Blood samples were collected every 10 days from each bear, from October through to mid-April. The collection dates encompassed an active pre-hibernation period, a disuse hibernation period and a post-hibernation remobilization period. Hibernation began in early January and ended in early April.

Radioimmunoassays were performed to determine the serum concentrations of PICP (bone formation marker), ICTP (bone resorption marker) and cortisol. Reagents for the serum ICTP and PICP assays were obtained from Diasorin (Stillwater, MN, USA). 100 μl serum samples were run in duplicate. The intra-assay coefficient of variation were 4.8% for ICTP and 2.8% for PICP. The serum cortisol levels were determined using reagents from Diagnostic Products Corporation (Los Angeles, CA). 50 μl serum samples were run in duplicate; the intra-assay coefficient of variation was 5%. Enzyme-linked immunosorbant assay (ELISA) was performed to determine the serum concentrations of NTX (bone resorption marker) using reagents from Ostex International (Seattle, WA, USA). 50 μl serum samples were run in duplicate; the intra-assay coefficient of variation was 4.6%. For all four assays,

measurements were repeated on samples with an intra-sample coefficient of variation greater than 10%.

For each of the five bears, a mean value for each period (pre-hibernation, hibernation and post-hibernation) was determined for PICP, ICTP, NTX and cortisol. For each bear there were ten pre-hibernation samples, nine hibernation samples, and one post-hibernation sample. One-way analyses of variance (ANOVA) were used to compare the mean (N=5) serum levels of PICP, ICTP, NTX and cortisol between the three study periods. Significant ANOVA values were followed up with Fisher's PLSD tests for multiple-means comparisons. Linear regressions were performed to assess correlations between cortisol and ICTP and cortisol and PICP to assess the potential role of cortisol in mediating bone remodeling. A significance level of 0.05 was used for all statistical analyses.

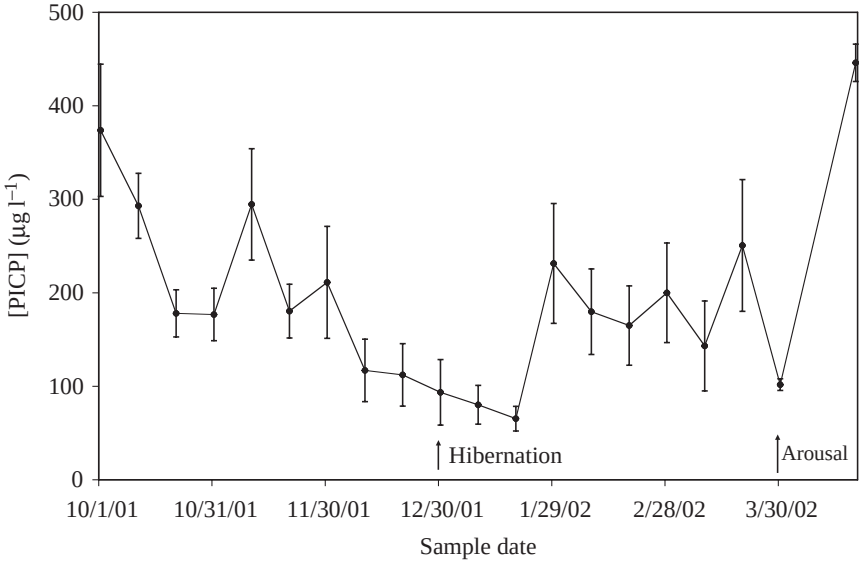

Fig. 1. Seasonal variations in the serum concentration of the bone formation marker type 1 collagen carboxy-terminal propeptide (PICP); values are means ± S.E.M. (N=5). The arrows indicate the beginning and end of hibernation.

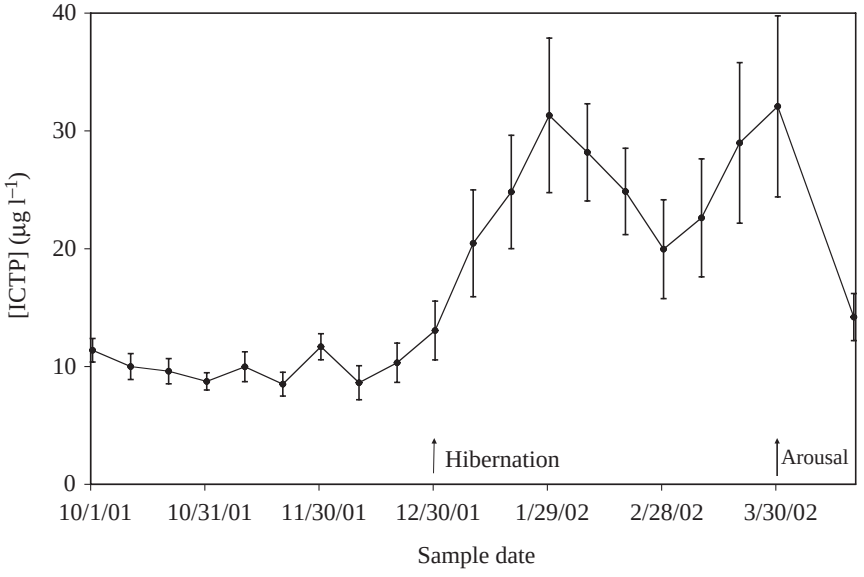

Fig. 2. Seasonal variations in the serum concentration of the bone resorption marker type 1 collagen carboxy-terminal telopeptide (ICTP); values are means ± S.E.M. (N=5). The arrows indicate the beginning and end of hibernation.

Results

During the active period prior to hibernation the concentration of the bone formation marker PICP showed a trend of continually decreasing values (Fig. 1). However, early in the hibernation period the PICP concentration increased and remained elevated for the remainder of hibernation. 2 weeks after arousal from hibernation, the concentration of PICP spiked to its highest level over the 6-month study period.

The bone resorption marker ICTP showed a sustained increase during the hibernation period (Fig. 2). In the 3-month pre-hibernation period the mean ICTP concentration showed very little fluctuation, remaining between 9 and 13 μg l^{-1}. Within the first 2 weeks of the hibernation period, mean ICTP levels increased to 20 μg l^{-1} and remained elevated for the duration of hibernation. 2 weeks after arousal, ICTP levels dropped rapidly towards pre-hibernation values. Unlike ICTP, the serum concentration of the bone resorption marker NTX did not show seasonal variations (Fig. 3). The serum concentration of cortisol was greatest during the hibernation period and dropped following arousal from hibernation, similar to ICTP concentration (Fig. 4). However, cortisol showed greater variability than ICTP.

There was no significant (P=0.207) difference in the mean concentration of PICP between the pre-hibernation period (203±28 μg l^{-1}) and the hibernation period (158±21 μg l^{-1}) (Fig. 5). However, the mean concentration of ICTP significantly (P=0.0028) increased by more than twofold during the hibernation period (26±3.9 μg l^{-1}) compared to the pre-hibernation period (10±1.1 μg l^{-1}) (Fig. 6). This finding suggests that bone formation was uncoupled from bone resorption, resulting in a net bone loss during disuse. However, the mean values of serum NTX concentration did not significantly (P=0.7215) change during hibernation compared to the pre-hibernation period (Fig. 7). The discrepancy in seasonal variations in the levels of bone resorption markers ICTP and NTX is probably due to

the way the bone collagen was broken down and is addressed in detail in the discussion.

During the remobilization period, 2 weeks after arousal from hibernation, the concentration of the bone formation marker PICP significantly (P=0.0001) increased over pre-hibernation and hibernation values (Fig. 5). This finding suggests that bone formation rapidly increased during remobilization. 2 weeks into the post-hibernation period the ICTP concentration had returned to pre-hibernation levels (Fig. 6). Taken together, these findings suggest that immediately upon remobilization, bears begin to replace any bone that was lost during hibernation.

Serum concentrations of cortisol showed similar behavior to ICTP, significantly (P=0.0073) increasing during hibernation and returning to pre-hibernation values during remobilization (Fig. 8). Regression analysis showed a significant (P=0.0236), but weak (r^2=0.254) negative correlation between serum cortisol and PICP levels. Serum ICTP concentration showed a significant (P=0.0062), but weak (r^2=0.348) positive correlation with serum cortisol.

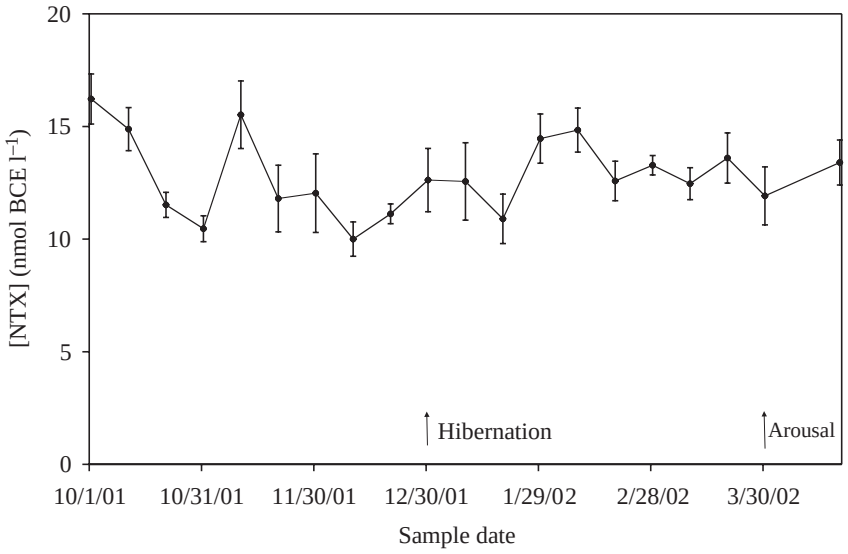

Fig. 3. Seasonal variations in the serum concentration of the bone resorption marker type 1 collagen amino-terminal telopeptide (NTX); values are means ± S.E.M. (N=5). The arrows indicate the beginning and end of hibernation. BCE, bone collagen equivalents.

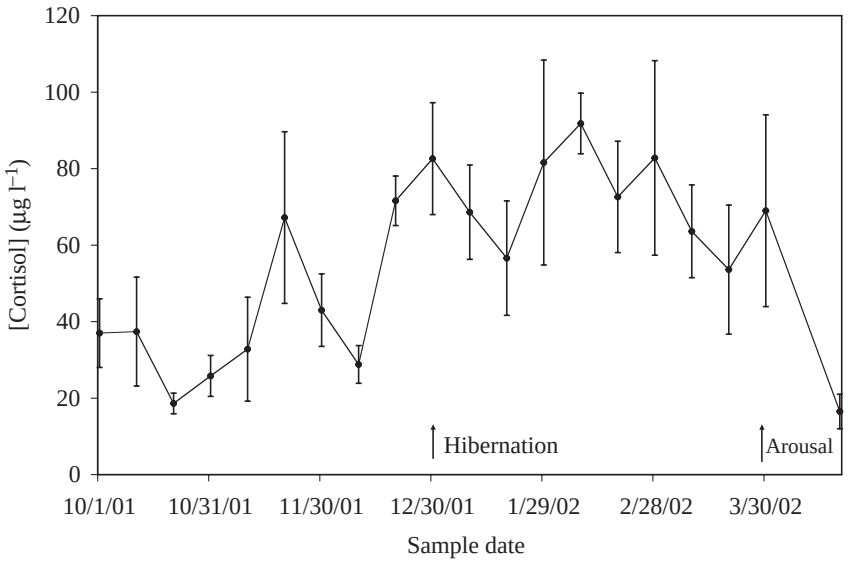

Fig. 4. Seasonal variations in the serum concentration of cortisol; values are means ± S.E.M. (N=5). The arrows indicate the beginning and end of hibernation.

Discussion

The findings of the present study on the time course of bone remodeling during inactivity in captive black bears corroborate the results of our previous study on wild bears in which serum markers of metabolism were analyzed for single time points in active and hibernating periods (Donahue et al., 2003). In the present study we show that bone resorption probably remains elevated over the entire hibernation period compared to the active period and that bone formation is not likely to be impaired by hibernation, as indicated by the levels of serum markers of bone remodeling. This is in contrast to other animals that lose bone during inactivity as a result of both increased bone resorption and decreased bone formation. Our data support the hypothesis that black bears have evolved a unique regulatory mechanism that minimizes bone loss during the disuse period of hibernation. The findings in the present study on the bone-formation marker measurements suggest that the bone lost during disuse, by increased bone resorption, may be quickly recovered as a result of increased bone formation in the spring and may remain elevated until the bone mass is fully restored (Fig. 1).

Notwithstanding these provocative data, some important limitations to our observations need to be considered. First, because of the limited number of post-hibernation samples, it is uncertain if, and for how long, bone formation is elevated following hibernation. In our previous study we measured serum PICP in 17 wild black bears over the course of the summer, although only one sample was taken from each bear (Donahue et al., 2003). In that previous study, we found that serum PICP levels were 4–5-fold higher in early to mid-June than during hibernation; in July and August PICP concentrations were similar to hibernation values. Given the results of these two studies, it is reasonable to hypothesize that bone formation is higher in black bears in the first 2–3 months following hibernation than during

the remaining portion of their physically active life and during hibernation. This hypothesis is supported by the histological data of Floyd et al. (1990), which showed that the bone formation rate in black bears was several-fold higher in the spring than during hibernation. A more thorough analysis of the post-hibernation bone formation indicators is clearly needed to further support this hypothesis. Second, serum markers of bone metabolism reflect bone remodeling in the entire skeleton. Therefore, it is unclear if all bones behave similarly. Third, there is a discrepancy between the seasonal variations in serum ICTP and NTX in black bears. The rise in ICTP indicates that bone resorption is increased during hibernation, but the unchanged level of NTX suggests that bone resorption is unchanged. However, it has been shown that different enzymes that degrade type I bone collagen produce different epitopes of the type I collagen telopeptide fragments (ICTP and NTX) (Atley et al., 2000; Garnero et al., 2003). Bone resorption by the proteinase cathepsin K produces NTX fragments (Atley et al., 2000); resorption by matrix metalloproteinases produces ICTP fragments (Garnero et al., 2003). Thus, it is likely that bone resorption is increased in hibernating bears by increased matrix metalloproteinase activity.

Bone remodeling has been studied in other hibernating animals, most notably in ground squirrels, golden hamsters and little brown bats (Haller and Zimny, 1977; Kwiecinski et al., 1987; Steinberg et al., 1979, 1981, 1986). These studies suggest that bone is lost during hibernation by reduced osteoblastic formation and increased bone resorption. Bone resorption is believed to occur in these animals by osteocytic osteolysis, although intracortical osteoclastic resorption cavities have been observed post-hibernation in little brown bats *Myotis lucifugus* (Kwiecinski et al., 1987). In little brown bats bone loss is manifest by reduced cortical thickness and mineral density at the end of hibernation compared to the beginning of hibernation (Kwiecinski et al., 1987). Little brown bats also have significantly higher plasma calcium levels in some hibernation months compared to the active summer months, unlike black bears, which show no significant difference in plasma calcium levels between active and hibernation periods (Floyd et al., 1990). This discrepancy in seasonal plasma calcium levels may be due to the observation that osteoblastic bone formation is arrested during hibernation in other animals (Steinberg et al., 1986), whereas black bears possibly prevent hypercalcemia during hibernation by maintaining osteoblastic bone formation, as our data and those of Floyd et al. (1990) suggest.

Both little brown bats and black bears mate prior to hibernation and have delayed implantation (Hellgren, 1998; Kwiecinski et al., 1991). However, black bears give birth and nurse their cubs during hibernation, while gestation and lactation in little brown bats occurs in the summer after hibernation. During pregnancy and lactation, calcium is liberated from bones (Cross et al., 1995). The additional burden of pregnancy and lactation on bones during hibernation may explain, at least in part, the disproportionately small birth weight of black bears compared to little brown bats. At birth, black bears only weigh about 0.3% of their mother's mass, while little brown bats are about 30% of their mother's mass (Kwiecinski et al., 1987; Oftedal et al., 1993). However, despite this additional burden of pregnancy and lactation on the bone's calcium supply, female black bears may be able to recover bone during the active summer months as well as males can. We previously found that bone strength and mineral content were significantly higher in female bears than in male bears near the end of their active period (i.e. October) (Harvey and Donahue 2003). Female black bears possibly attain a higher bone mineral content than males during the active

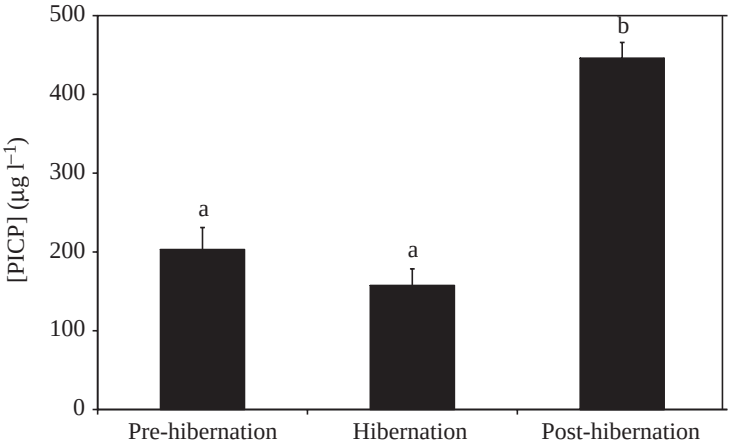

Fig. 5. Mean serum PICP concentrations for the three study periods. Bar values are means ± S.E.M. (*N*=5). Groups with the same letter are not significantly different from each other. The PICP concentration was not significantly different during hibernation than during the pre-hibernation period. However, post-hibernation, PICP concentration was significantly higher than in the other two periods.

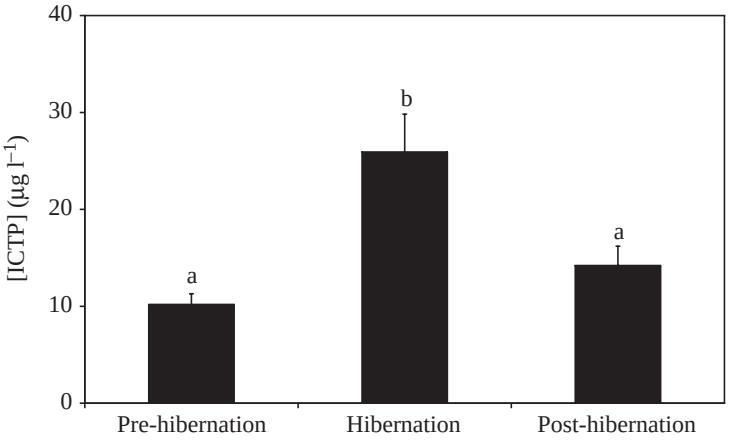

Fig. 6. Mean serum ICTP concentrations for the three study periods. Bar values are means ± S.E.M. (*N*=5). Groups with the same letter are not significantly different from each other. The serum ICTP concentration significantly increased during hibernation and returned to pre-hibernation values upon remobilization.

summer months, in preparation for the additional burden of pregnancy and lactation during hibernation. Furthermore, we previously found that bone strength, mineral content and porosity did not change with age in either gender. These findings suggest that the bone lost during annual hibernation periods, due to increased resorption, can be recovered annually by bears during the active summer months.

In humans, high serum concentrations of cortisol, as well as glucocorticoid therapy, are associated with increased bone resorption and decreased bone formation, which decrease bone mass and increase the incidence of spontaneous fracture (Libanati and Baylink, 1992). Additionally, a significant negative correlation between serum cortisol levels and bone mineral density has been shown in men (Dennison et al., 1999). These findings suggest a role for cortisol in the regulation of bone turnover in hibernating bears. However, it is unclear why the increased serum cortisol during hibernation did not significantly change bone formation. One possible explanation is that other factors influence the metabolic activity of bone-forming osteoblasts. The hormone leptin is possibly involved in the regulation of bone metabolism during hibernation, since leptin is an appetite and bone-formation suppressor, although its effects on bone metabolism may be secondary to its effect on appetite suppression. Steep and continuously rising leptin levels, beginning about 1 month prior to hibernation, have been reported for European brown bears, coincident with the period when bears become anorectic (Hissa et al., 1998). We also noted in our study that the bears stopped eating in December shortly before hibernating, despite the availability of food. Interestingly, our PICP data show an apparent negative correlation with the data of Hissa et al. (1998): pre-hibernation, bone formation is low and leptin levels are high; at arousal, bone formation is high and leptin levels low. Thus, when hibernating bears emerge from their winter dens, decreased serum leptin levels would permit increases in bone formation, allowing the bears to recover the bone lost during hibernation (due to increased resorption).

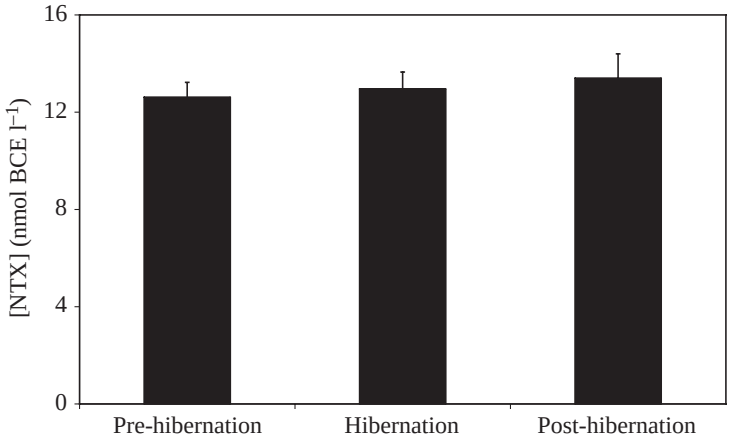

Fig. 7. Mean serum NTX concentrations for the three study periods. Bar values are means ± S.E.M. (*N*=5). Serum NTX concentration was not significantly different between the three study periods. BCE, bone collagen equivalents.

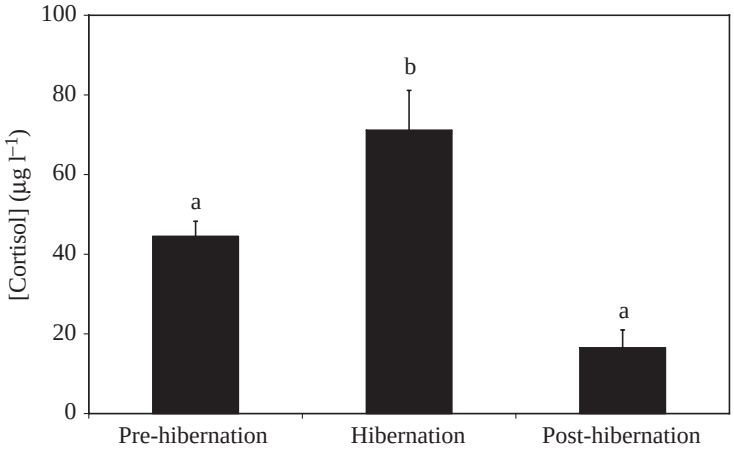

Fig. 8. Mean serum cortisol concentrations for the three study periods. Bar values are means ± S.E.M. (*N*=5). Groups with the same letter are not significantly different from each other. The serum ICTP concentration significantly increased during hibernation and returned to pre-hibernation values upon remobilization.

The question we set out to answer was, how can black bears maintain bone mass and strength when annual hibernation (i.e. disuse) and active periods are approximately equal (i.e. 6 months)? Our results suggest the answer to be that bears maintain their bone mass by maintaining normal bone formation during disuse and by rapidly increasing bone formation during remobilization to recover the bone lost by increased bone resorption during hibernation. However, further investigation is need to substantiate this hypothesis. A new, and perhaps equally intriguing question is, what are the biological mechanisms that regulate bone remodeling in hibernating black bears? Are parathyroid hormone, calcitonin, and leptin involved? It is known that hormones, including PTH, sensitize bone cells to mechanical stimulation *in vitro* (Ryder and Duncan, 2000; Sekiya et al., 1999). Thus, one possible way of elevating bone formation following hibernation is the sensitization of bone-forming osteoblasts, by circulating hormone levels, during remobilization after spring arousal.

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