

Maintenance of bone mass and architecture in denning black bears (*Ursus americanus*)

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Abstract

Bone mass is dramatically compromised during periods of weightlessness, inactivity, or bed rest. Animals that hibernate reduce their body temperature, heart rate and metabolic activity, and likewise lower bone turnover activity during their immobile state. Black bears *Ursus americanus*, however, do not hibernate, but rather overwinter by denning during which they maintain a nearly normal functional heart rate, cardiac output and temperature. Furthermore, markers of bone turnover in black bears are maintained during denning periods. Thus, the purpose of this work was to determine if the denning state of relative immobility in black bears results in changes in bone mass and bone architectural structure. Harvested forelimbs (ulna and radius) were compared between pre- and post-denning black bears using X-ray imaging, dual energy X-ray absorptiometry, and micro-computed tomography to quantify total distal forelimb bone mineral density, cancellous bone mineral density, bone mineral content, bone volume fraction, degree of anisotropy, structure model index, and trabecular thickness. No significant differences in any of the measured parameters were found in comparing radius and ulna from autumn and spring bears in this cross-sectional sample, suggesting that black bears did not experience a significant change in bone mass or architecture during denning. The statistical power for detecting a significant difference ($P \leq 0.05$) for this sample was 0.8. The specific mechanism by which the preservation of bone was attained may be related to skeletal muscle interaction or circulating systemic hormones.

Key words: bone mass, black bear, *Ursus americanus*, micro-computed tomography, bone architecture, osteopenia, denning

INTRODUCTION

Periods of inactivity and disuse produce osteopenia when osteoclastic resorption exceeds osteoblastic formation of bone (Aaron, Makins & Sagreiya, 1987; Mundy, 1987; Floyd, Nelson & Wynne, 1990). Net bone loss and alterations in the cancellous bone architecture have been observed in bedridden patients (Chantraine, Nussgens & Lapiere, 1986), paralysed patients (Deitrick, Whedon & Shorr, 1948), inactive elderly (Simkin, Ayalon & Leichter, 1987), and immobilized animals (Deitrick *et al.*, 1948; Cann, Adachi & Holton, 1980). Experimental models of disuse, microgravity and immobilization in animals have demonstrated that marked reductions in mechanical stimuli have dramatic effects on the mechanical and physical properties of bone (Anderson & Cohn, 1985; Mundy, 1987; Raisz, 1988; Jee & Ma, 1999).

A comparable result might be expected in denning animals. Some animals hibernate and lower their body temperature, cardiac output and metabolism. Black bears *Ursus americanus*, however, den for 5–7 months of the year, and during that time they do not eat, drink, urinate or defecate, but maintain a functional heart rate, cardiac output and temperature (Nelson *et al.*, 1983). During denning, the heart rate of a black bear declines from an average of 155 beats/min to 10 beats/min, and consequently their metabolism slows by half to save energy (Fair & Rogers, 1990). Serum calcium concentrations, however, are maintained during denning (Floyd *et al.*, 1990).

Black bears are not classic hibernators because they have adapted the ability to maintain high body temperatures in the face of frigid climates, and conversely they are capable of sleeping continuously throughout their denning period (Lundberg *et al.*, 1976). During denning, black bears are anuric, and have no means of removing the excess calcium excreted in the kidneys. None the less, serum calcium concentration during denning and non-denning periods remains constant (Clouston & Lloyd,

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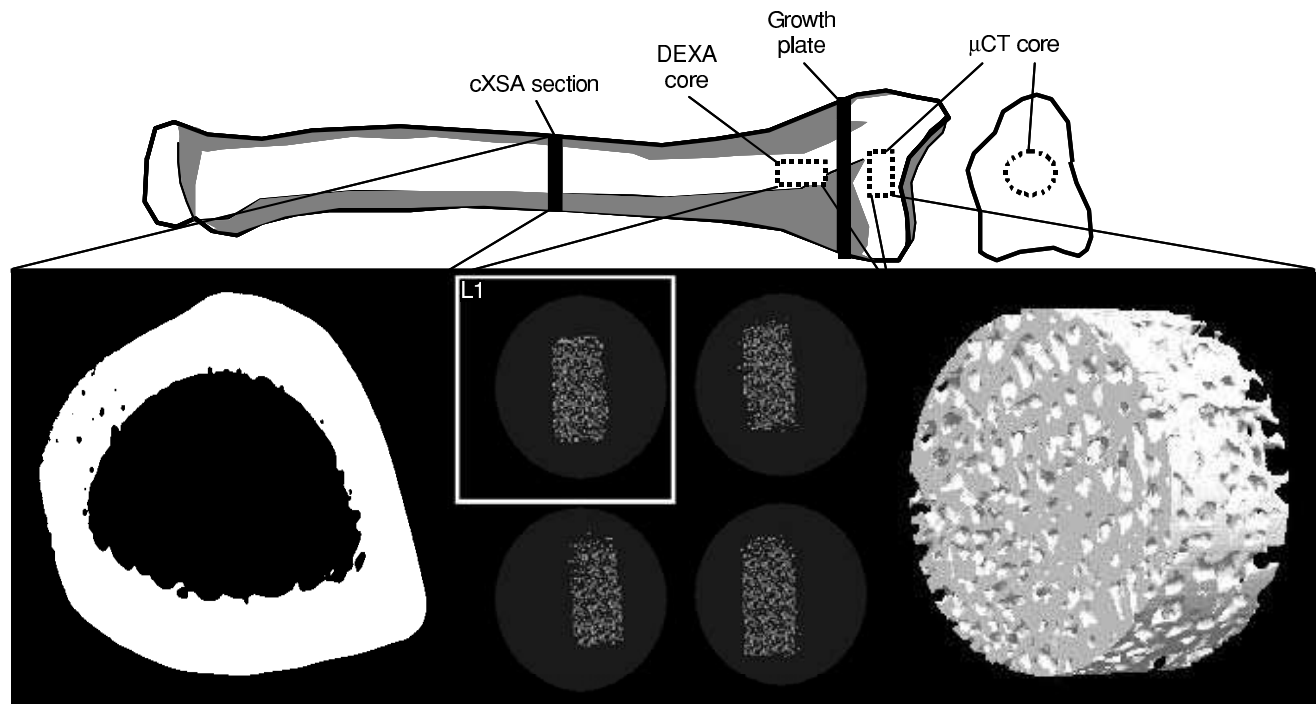


Fig. 1. Sections for measuring cortical cross-sectional area (cXSA) (mm^2), cancellous bone mineral density (DEXA) (g/cm^2) and micro-computed tomography (μCT).

1987), suggesting that calcium is being re-incorporated into the musculoskeletal system (David, Nelson & Alt, 1988). Harlow and colleagues (2001) found that denning bears lost $< 23\%$ of their muscular peak force with no loss of skeletal muscle cell number or size over 130–170 days of inactivity. Black bears do not exhibit the expected trend of muscle atrophy associated with prolonged disuse, but conservation of calcium and skeletal muscle relies heavily on the skeleton. During the general unloading of the skeleton and apparent reduction in mechanical stimuli during the winter denning months, one would postulate that bone mineral density and content would decrease, and cancellous bone architecture would significantly change. Thus, the purpose of this cross-sectional study was to assess whether black bears were able to maintain their bone mass and architecture during their winter denning period.

METHODS AND MATERIALS

Experimental animals

In strict compliance with Wildlife Management Department of Alberta Sustainable Resource Development bears were killed by provincially licensed hunters during the official hunting season. The pre- and post-denning bears were collected in 1 hunting season (2000). Culled spring (post-denning) bears were mobile for an average of 2 weeks post-denning and all autumn (pre-denning) bears were collected during September, just before denning. The authors did not have contact with any live animals for

the purpose of this study, and the participating licensed hunters agreed to donate forelimb samples from their kills. Right and left forelimb bones (radius and ulna) were harvested from the carcasses, frozen and sent to the authors' institution. The ulna and radius were harvested from pre-denning (autumn: male, $n = 17$; female, $n = 5$) and post-denning (spring: male, $n = 8$; female, $n = 15$) black bears. All soft tissue was removed, and the harvested forelimbs were stored at -40°C . Pre-denning (autumn) animals averaged 4.5 years and the post-denning cohort averaged 5.4 years.

Bone properties of the black bear forelimbs were quantified at both the macro- and micro-structural levels. At the macro-structural level, body mass (BM) (kg), diaphyseal morphology (cXSA), total distal bone mineral density (dBMD), cancellous bone mineral density (cBMD), and bone mineral content (BMC) were assessed for all bone samples ($n = 45$) (Fig. 1). At the micro-structural level, 8 bears (4 from spring and 4 from autumn), all of similar body mass, were selected for micro-computed tomography (μCT) imaging and morphological analysis (Fig. 1). Those analyses yielded trabecular bone architectural indices of bone volume ratio (bone volume with the sample/total volume of the sample) (BV/TV), structure model index (SMI), degree of anisotropy (DA), and trabecular thickness (Tb.Th).

Bone mineral density

Total distal forelimb bone mineral density (dBMD) was determined by dual energy X-ray absorptiometry (DEXA)

scans of the radius and ulna using a standard human clinical forearm scanning protocol (Hologic 2000, Hologic, Waltham, MA). To evaluate changes in cancellous bone mineral density (cBMD), cancellous bone cores (5 mm diameter, 8 mm length) were removed from the primary spongiosa of the distal radius, just distal to the growth plate from the central region of the distal radius (Fig. 1). Cores were scanned using the Hologic small animal protocol (Hologic, Waltham, MA).

Diaphyseal morphology

Diaphyseal morphology was evaluated using 0.5 mm thick wafers; cut from the mid-radial diaphysis using a Emco Unimat diamond wafer saw (Fig. 1). These slices were then scanned by X-ray (Faxitron 43855A, Faxitron X-ray Corporation, Buffalo Grove, IL), and the resulting images were converted to Tag Image File Format (TIFF) images using Camscan software on a Masterscan Interpretive Densitometer (CSPI Inc. Billerica, MA). The TIFF images were then used to calculate cortical cross-sectional area (cXSA) (mm^2) using a customized program, based on image analysis routines from Matlab v.6.0.

Cancellous bone mineral content

The mineral content of the cancellous bone cores was determined by ashing. The cores were dried for 48 h at 100 °C (Thermolyne F62700, Barnstead/Thermolyne Corporation, Dubuque, IA) and assessed for dry mass. The cores were then ashed for 48 h (600 °C) and measured for mineral ash mass. Mineral content was calculated as the percentage of mineral ash mass per dry bone mass.

Cancellous bone micro-architecture

Micro-architectural measures were obtained by removing and scanning 16 cores (left and right from the radii of each of the 4 spring and 4 autumn bears). Each core (10-mm diameter) was harvested from the secondary spongiosa of the distal radius between the cortical subchondral plate and growth plate (Fig. 1). Each specimen was scanned (SkyScan 1072 Desktop X-ray μ CT-scanner, Aartselaar, Belgium) with an exposure time of 3.6 s, rotation step of 0.45°, frame averaging of 2, and gain of 1, producing a nominal isotropic resolution of 18.23 $\mu\text{m}/\text{pixel}$. A global threshold was used on the resulting image data to produce segmented images representing the cancellous bone structure (SkyScan 2D-Cone-Beam CT). The same threshold parameters were used for all samples. From the segmented data for each of the 16 cores, a region of interest was selected 15 slices (273 μm) above the growth plate so as to include only secondary cancellous bone (Fig. 1). The image data were reconstructed into 3-D isosurfaces of cancellous bone (Fig. 1). From these 3-D reconstructions, bone volume ratio

Table 1. Morphometry and bone mineral content of pre-denning (autumn) and post-denning (spring) black bears *Ursus americanus*. Values are means $\pm$ SD. BM, body mass; BMC, bone mineral content; cXSA, cortical cross-sectional area; BV/TV, bone volume to total volume ratio; SMI, structure model index; DA, degree of anisotropy; Tb.Th, trabecular thickness

Measure	Autumn	Spring	P-value
BM (kg) ^a	243.3 $\pm$ 88.2	201.9 $\pm$ 85.8	0.176
BMC (g) ^a	37.8 $\pm$ 5.8	38.9 $\pm$ 4.9	0.211
CXSA (mm^2) ^a	98.9 $\pm$ 27.8	107.5 $\pm$ 13.7	0.332
BV/TV ^b	40.6 $\pm$ 3.6	41.9 $\pm$ 6.6	0.620
SMI ^b	0.40 $\pm$ 0.18	0.39 $\pm$ 0.18	0.946
DA ^b	0.18 $\pm$ 0.01	0.20 $\pm$ 0.05	0.276
Tb.Th (mm) ^b	0.15 $\pm$ 0.02	0.16 $\pm$ 0.02	0.956

^a Performed using all specimens ($n = 45$).

^b Morphological assessment was executed on a sub-set of specimens ($n = 8$).

(BV/TV), degree of anisotropy (DA), trabecular thickness (Tb.Th), and structure model index (SMI) were obtained with commercially available algorithms (SkyScan 3-D ANT).

SMI (Table 1) is a non-metric representation of 3-D sections of trabecular structure. SMI ranges from 0 indicating a plate-like trabecular structure to 3 indicating a rod-like structure (Hildebrand & Rueggsegger, 1997). The DA (Table 1) was a measure of organization and distribution of cancellous bone.

Statistics

To gauge the capability of black bears to maintain bone mass and architecture throughout the denning period, animals harvested from pre-denning (autumn: male, $n = 17$; female, $n = 5$) and post-denning (spring: male, $n = 8$; female, $n = 15$) were respectively divided into autumn and spring cohorts. Full sample sets ($n = 45$) were used for measures of BW, BMD, BMC and cXSA. For the cancellous microstructure analysis, average left and right data, from 8 animals (4 autumn, 4 spring), were used for morphometric evaluations. All results were analysed using a Mann–Whitney non-parametric analysis (SPSS for Windows 11.01, SPSS Inc., Chicago, IL) to determine significant differences between autumn and spring cohorts. All data are reported as means $\pm$ SD.

RESULTS

No statistically significant differences ($P \leq 0.05$) were found between the autumn and spring bears for BW, BMD (Fig. 2), BMC and morphometric measures (Table 1). The cross-sectional area of the mid radial cortical bone was also calculated using a customized Matlab program, and as with other measures, there were no statistically significance differences (Table 1).

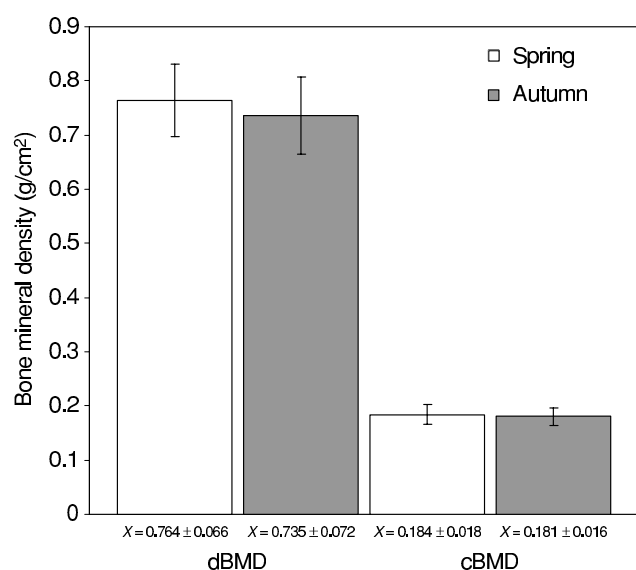


Fig. 2. Comparison of total forelimb (dBMD) and cancellous (cBMD) bone mineral density between spring (post-denning) and autumn (pre-denning) cohorts of *Ursus americanus*.

DISCUSSION

The aim of the present study was to determine whether black bears were capable of maintaining bone mass and architecture throughout winter denning despite an apparently dramatic reduction in limb loading. Our cross-sectional results suggested that radial and ulnar bone mass and trabecular bone architecture were not significantly different between the spring and autumn cohorts, which were differentiated by the denning period. The precise osteoregulatory mechanisms for preserving bone during denning remain elusive, but recent complementary data involving skeletal muscle shivering and circulating leptin (a hormone) have suggested potential mechanisms.

The osteoregulatory mechanism(s) in black bears that allow them to maintain bone mass during denning are essential for their survival. If black bears responded similarly to humans during periods of inactivity (such as paraplegics with a 33% loss of iliac trabecular bone mass over 25 weeks; Minaire *et al.*, 1974), their skeleton would be severely compromised after denning (Chantraine *et al.*, 1986). That black bears readily ambulate and forage upon emerging from their dens suggests that the bears would require a unique osteoregulatory mechanism to maintain sufficient weight-bearing bone mass. Along with mineralization at the macro-structural level, cortical bone xCSA was preserved. The maintenance of xCSA is imperative to sustaining the functional capacity of the skeletal system of black bears. The data from the current study further support the evidence suggesting that the bears do indeed have some mechanism to maintain bone mass during non-weight bearing periods of denning.

At the macro-structural level, the lack of significant differences between the spring and autumn cohorts in dBMD, cBMD, and BMC, indicated that mineral content

and density were maintained over the denning cycle. Maintenance of mineral status, especially calcium, was significant when considering the eucalcaemic and anuric behaviour of denning black bears. Floyd *et al.* (1990) found that denning black bears maintained a constant plasma $[Ca^{2+}]$, eucalcaemia, but did not urinate or defecate while denning and, therefore, had no means of eliminating excess serum Ca^{2+} if osteoclastic activity surpassed osteoblastic activity (Floyd *et al.*, 1990). If the rate of bone resorption exceeded the rate of bone deposition, as is typical with inactivity, serum calcium levels would climb. Similar to black bear body metabolism, markers of bone turnover are maintained during denning (Floyd *et al.*, 1990). To avoid hypercalcaemia in an anuric state, the calcium must be stored back in the bone. Yet bone formation during the relative inactivity of denning occurs in an environment with dramatically reduced mechanical stimuli.

The trabecular orientation and anisotropy in peri-articular cancellous bone varies throughout the joint and is influenced by the predominant local mechanical milieu (Goldstein, 1987; Keaveny *et al.*, 2001). Furthermore, changing the joint loading patterns can alter the trabecular architecture (Boyd, Müller & Zernicke, 2002). Though black bears may maintain bone mass to ensure eucalcaemia in an anuric state, the apparent lack of mechanical stimuli in denning might have resulted in an altered trabecular orientation between the autumn (pre-denning) and spring (post-denning) bears. The data from the current study, however, suggest that trabecular architecture is maintained.

By comparing the values between the autumn and spring bears it was obvious that there was no significant loss of cancellous bone mass. Not only did black bears conserve the volume of cancellous bone, but they also conserved bone structure. As the black bears were inactive and, thus, were exposed to a global unloading or shift in the loading pattern of the skeletal system, it would be expected that the distribution and shape of trabeculae would change and become less organized. The structure model index is a measure of the shape of the plate-like vs the rod-like trabeculae. In clinical osteoporosis, trabeculae tend to change from plate-like to rod-like (Ding & Hvid, 2000) owing to perforations in the plate structures with excessive resorption. The plate-like trabecular structure was maintained for the spring cohort of black bears. Maintaining a plate-like structure would be advantageous because rod-like structures are more susceptible to large deformations, such as bending and rotation of trabeculae, than plate-like trabeculae (Morgan & Keaveny, 2001). By maintaining a more plate-like trabecular structure the bears reduced their risk of fracture following denning. Changes in the trabecular organization did not occur either. There was no difference in the degree of anisotropy (DA) from the autumn to spring bears. Thus, not only were the volume and shape of the cancellous bone preserved, but the distribution and orientation of trabeculae were also maintained.

A lack of significant differences between spring and autumn cohorts suggests that an osteoregulatory

mechanism exists during the denning period of black bears. Recent findings point to skeletal muscle shivering or hormones (e.g. leptin) as candidate factors in the preservation of bone mass and structure during denning in bears (Harlow *et al.*, 2001; Ozata, 2002).

During the duration of denning, it would be expected that skeletal muscle would atrophy significantly. Denning black bears, however, seem to lose < 23% of their strength over 130 days, unlike humans, who have been predicted to be weakened by up to 90% over a similar 130-day period (Harlow *et al.*, 2001). In humans, the loss of strength with disuse was associated with a decrease in the number and size of muscle fibres, but in muscle biopsies taken from black bears during early and late winter, no loss of skeletal muscle cell number or size was found (Harlow *et al.*, 2001). Muscle strength may be maintained by rhythmic stimulation of the muscles with shivering (high frequency muscle contractions) through the denning period (Harlow *et al.*, 2001). If shivering was partially responsible for the preservation of muscle protein and associated strength conservation, then shivering may also be sufficient to produce important mechanical stimuli to suppress loss of bone mass associated with disuse. It has been proposed that the anabolic activity of bone, suppressed by disuse, is normalized by exposure to extremely low-magnitude mechanical stimuli (Rubin, Xu & Judex, 2001; Judex, Donahue & Rubin, 2002). Over a 28-day period, 10 min/day of a 90 Hz stimulation increased bone formation rate by 97% in the proximal tibia of adult female rats (Rubin *et al.*, 2001). Thus, shivering in skeletal muscles of denning black bears may provide adequate mechanical stimuli to preserve bone mass and maintain trabecular micro-architecture.

Black bears monitored during denning maintained regular metabolic activities including functional heart rate, cardiac output, and body temperature (Floyd *et al.*, 1990). The metabolic activity required 4000 kcal/day (Floyd *et al.*, 1990), which was facilitated by the burning of stored adipose tissue. Before denning, the protein hormone leptin is accumulated within new adipose tissue deposits, and the systemic concentration of leptin is accumulated with this seasonal increase in adipose tissue. Leptin levels are proportional to the amount of energy stored as adipose tissue as well as the state of energy balance, such that fasting results in a decrease in leptin, while overfeeding increases leptin levels (Ozata, 2002). Therefore, higher levels of adipose tissue, as seen in pre-denning black bears, were found to be consistent with increased serum leptin (Holloway *et al.*, 2002). *In vitro* exposure of mouse mandibular chondrocytes to leptin produced enhanced proliferation in osteoblastic cells, while significantly stimulating bone mineralization (Maor *et al.*, 2002). Additionally, the administration of leptin to mice led to a significant increase in femoral length, total body bone area, both cortical and cancellous, as well as increases in BMD and BMC (Gordeladze *et al.*, 2002). Leptin not only had an anabolic effect, but was also shown to play a role in osteoclast inhibition in human stromal cells (Burguera *et al.*, 2001). Hence, leptin may play a significant role in enhancing osteoblastic

activity and also in inhibiting osteoclastic activity. The pathway through which leptin acts to preserve bone mass, either by promoting osteoblastic activity or inhibiting osteoclastic activity, is not entirely known. It has been suggested, however, that leptin does not act directly on osteoblasts, but rather through a central pathway following binding to its specific receptors located on the hypothalamic nuclei (Amling *et al.*, 2001). The nature of the signal downstream of the hypothalamus is currently under investigation. Throughout denning, stored fat not only facilitates homeostatic metabolism but may also advantageously aid in the preservation of bone mass linked to elevated serum leptin levels.

The data from the current study demonstrated that black bears *Ursus americanus* were able to maintain their bone mass and trabecular architecture over a period of denning, averaging 130 days. These data suggest that black bears have adapted to prevent bone loss and changes generally associated with extended periods of physical inactivity. Potentially, skeletal muscle shivering or circulating leptin levels may be related to the preservation of skeletal bone mass during denning.

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