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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

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Abstract

Disuse typically uncouples bone formation from resorption, leading to bone loss which compromises bone mechanical properties and increases the risk of bone fracture. Previous studies suggest that bears can prevent bone loss during long periods of disuse (hibernation), but small sample sizes have limited the conclusions that can be drawn regarding the effects of hibernation on bone structure and strength in bears. Here we quantified the effects of hibernation on structural, mineral, and mechanical properties of black bear (*Ursus americanus*) cortical bone by studying femurs from large groups of male and female bears (with wide age ranges) killed during pre-hibernation (fall) and post-hibernation (spring) periods. Bone properties that are affected by body mass (e.g. bone geometrical properties) tended to be larger in male compared to female bears. There were no differences ($p > 0.226$) in bone structure, mineral content, or mechanical properties between fall and spring bears. Bone geometrical properties differed by less than 5% and bone mechanical properties differed by less than 10% between fall and spring bears. Porosity (fall: $5.5 \pm 2.2\%$, spring: $4.8 \pm 1.6\%$) and ash fraction (fall: 0.694 ± 0.011 , spring: 0.696 ± 0.010) also showed no change ($p > 0.304$) between seasons. Statistical power was high ($>72\%$) for these analyses. Furthermore, bone geometrical properties and ash fraction (a measure of mineral content) increased with age and porosity decreased with age. These results support the idea that bears possess a biological mechanism to prevent disuse and age-related osteoporosis.

Keywords

cortical bone; mechanical properties; black bear; disuse osteoporosis

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Introduction

The skeleton is susceptible to bone loss induced by decreased mechanical loading. Disuse results in unbalanced bone remodeling and causes deleterious changes in bone structure and composition (Caillot-Augusseau et al., 1998; Li et al., 2005; David et al., 2006). Effects of disuse on cortical bone include increased intracortical porosity, decreased bone geometrical properties, decreased bone mineralization, and reduced bone strength (Gross and Rubin, 1995; Kaneps et al., 1997; Li et al., 2005). Hibernation is a natural animal model of disuse because hibernating animals greatly reduce physical activity to conserve energy (Nelson et al., 1973; French, 1985; Lariviere et al., 1994). Therefore, hibernating animals would be expected to lose bone during their seasonal dormancy.

Interestingly, hibernating bears may be uniquely resistant to disuse osteoporosis. Though previous studies using rats and dogs suggest that remobilization periods of 2–3 times the length of the disuse period are required for complete bone recovery (Kaneps et al., 1997; Weinreb et al., 1997), black bears undergo annual periods of disuse and remobilization that are approximately equal in length (5–7 months annually), but do not demonstrate increased intracortical porosity, decreased cortical bone geometry, or reduced cortical bone material properties with age, even near the end of their lifespan (Harvey and Donahue, 2004; Harvey et al., 2005; McGee et al., 2007a; McGee et al., 2007b). This suggests that bears either prevent bone loss during hibernation, or bears lose some bone during hibernation and are subsequently able to recover it at a faster rate than other animals.

Recent evidence suggests that bears fully prevent bone loss during hibernation. Grizzly bears experience decreased, but balanced, intracortical remodeling during hibernation which may help them to preserve cortical bone structure and strength. In fact, femurs from hibernating grizzly bears were less porous and more mineralized than femurs from age- and sex-matched active bears (McGee et al., 2008). These findings suggest that bears have evolved a unique biological mechanism to mitigate disuse-induced bone loss. However, previous studies of seasonal changes in bear bone properties were limited by a small sample size ($n \leq 4$ bears per season) and consequently, low statistical power. For example, the ultimate stress of grizzly bear femurs was not different between hibernating and active bears ($p = 0.2$), but the power to detect a physiologically relevant change in ultimate stress was low (42%) (McGee et al., 2008). Thus, it has not been conclusively demonstrated whether bears can prevent detrimental changes in cortical bone structure and strength during hibernation. Bones can be obtained in much larger quantities from wild black bears killed during hunting seasons, thus permitting more powerful analyses of the effects of hibernation on bone from bears in their natural environment. In this study cortical bone structure, mechanical properties, and mineralization were quantified in femurs from wild black bears (*Ursus americanus*) killed during pre- and post-hibernation periods. We hypothesized that post-hibernation bears would not demonstrate cortical bone loss (i.e., increased intracortical porosity, decreased bone mechanical properties and mineralization, or reduced bone geometrical properties) since bears maintain balanced bone remodeling during hibernation (Donahue et al., 2006; McGee et al., 2008).

Methods

Samples

One left or right femur was randomly selected from each of 65 black bears killed during the 2005 through 2007 fall and spring hunting seasons in Utah. Twenty-two were from bears killed in the fall (16 male, 6 female), and forty-three were from bears killed in the spring (31 male, 12 female). Ages were determined by the Utah Department of Wildlife Resources from the dental cementum annuli (Coy and Garshelis, 1992), and ranged from 1 to 19 years. Bears in Utah begin denning in late October and emerge in late April; the spring bears were killed

between April 26th and May 31st, and the fall bears were killed between August 26th and November 4th. Bones were cleaned of soft tissue and stored at -20 °C.

Whole bone bending

Femurs were thawed and rehydrated in water prior to mechanical testing. Bones were loaded to failure in three-point bending on an Instron mechanical testing system (Instron Model #8872, Canton, Massachusetts) using a crosshead speed of 1 mm/s and rounded supports ($r = 9.5$ mm), with the anterior side of the bone loaded in tension. The lower support span was adjusted to accommodate bones of different sizes as described previously (McGee et al., 2007a). The average length-to-depth ratio was 9.5 ± 0.6 . Ultimate load (P_u) was defined as the maximum force sustained before failure.

Geometrical properties

All bones fractured at the mid-diaphysis (i.e., midpoint of femoral length) beneath the loading fixture. Bones were reconstructed and the midshaft cross-sections were digitized as described previously (McGee et al., 2007a). Image analysis software (Scion Corporation, Frederick, Maryland) was used to calculate the periosteal area ($Ps.Ar$), cortical area ($Ct.Ar$), and endosteal area ($Es.Ar$) for each sample. A custom macro in Scion Image was used to calculate the cross-sectional moments of inertia for the mediolateral (bending) axis (I_{ML}) and anteroposterior axis (I_{AP}), product of inertia (I_p), maximum moment of inertia (I_{max}), centroid of the cross-section, neutral axis, and the x and y distances of the cortex location furthest from the neutral axis (Figure 1). Section modulus (SM) was calculated as I_{ML} divided by one-half of the outer (i.e., periosteal surface) anteroposterior diameter. Cortical thickness ($Ct.Th$) was calculated in 1 mm increments for the cross-section using image analysis software (Bioquant Osteo, Nashville, TN).

Whole bone mechanical properties

Beam bending theory was used to calculate the whole bone mechanical properties of each femur. Load data were converted to stress using Equation 1 (Levenston, 1995):

$$\sigma = \frac{P \cdot L \cdot (I_{AP} \cdot y - I_p \cdot x)}{4 \cdot (I_{ML} \cdot I_{AP} - I_p^2)} \quad (1)$$

where P was the load and L was the span between the lower supports. Ultimate stress (σ_u) was calculated from Equation 1, defining P as the ultimate load (P_u). Failure energy (U_f) was calculated as the area under the load-deformation curve up to fracture, and modulus of toughness (u) was calculated using Equation 2 (Turner and Burr, 2001):

$$u = \frac{U_f(3 \cdot c^2)}{I_{ML} \cdot L} \quad (2)$$

where c was one-half of the outer anteroposterior diameter.

Ash fraction

A 10 mm section of the diaphysis located immediately proximal to the reconstructed fracture section (i.e., 7.5 mm proximal to the midshaft) was removed and cleaned of marrow. The bone segments were dried at 100° C for 24 hours and ashed at 600° C for 48 hours. The ash fraction (a measure of mineral content) was calculated as the ash mass divided by the dry mass.

Porosity

A 15 mm section of the diaphysis located immediately distal to the reconstructed fracture section (i.e., 7.5 mm distal to the midshaft of the femur) was removed from each bone and histologically prepared. One section (70–90 μm thick) from each bone was stained in four increasing ethanol concentrations of a 1% basic fuchsin stain (70–100% ethanol) for 30 seconds each and rinsed in a 100% ethanol wash for 30 seconds. The sections were imaged at 40x magnification and the microstructure of the bone was analyzed using a software package (Bioquant Osteo). Porosity was defined as the ratio of porous area to bone tissue area, and included all porous spaces (vascular channels and remodeling cavities) except osteocyte lacunae and canaliculi.

Statistics

The primary hypothesis of interest was the test for differences in bone properties between fall and spring bears, after adjusting for possible age and sex effects. ANCOVA analyses were performed to compare bone properties between spring and fall bears, treating age and sex as covariates (5% level of significance). A failure to reject the null hypothesis does not necessarily demonstrate its veracity, so post-hoc power analyses were conducted to estimate the power to detect changes in bone properties that would be expected based on other animal models of disuse. The regression mean squared error was used as an estimate of model variance, and effect sizes thought to be meaningful responses to disuse in other animal models were determined from the literature (Table 1). The 95% confidence intervals for power were determined as functions of the confidence interval for the model variance and the value of the non-centrality parameter (Taylor and Muller, 1995; Rencher, 2000).

Results

Geometrical properties

For all bone geometrical properties examined, there were no differences between fall and spring bears ($p > 0.302$), yet geometrical properties significantly increased with age ($p < 0.0002$, $r > 0.339$) and differed between sexes ($p < 0.0007$) (Table 2). Bone geometrical properties were 17–57% larger in male compared to female bears. The 95% confidence interval for statistical power of detecting a 12.8% relative change (between pre- and post-hibernation) in cortical area ranged from 55% to 85%, with a calculated power of 72%. Similarly, the 95% confidence interval for statistical power of detecting a 9.4% relative change in cortical thickness ranged from 71% to 95%, with a calculated power of 86%.

Whole bone mechanical properties

Ultimate stress was not different between fall and spring bears ($p = 0.483$), but demonstrated an increasing trend with age ($p = 0.058$) (Table 2). Modulus of toughness also was not different between fall and spring bears ($p = 0.226$), but decreased with age ($p < 0.0001$) (Table 2). The decrease in modulus of toughness with age was likely related to the increase in ash fraction with age since the two variables were correlated ($p < 0.0001$, $r = -0.508$). Failure energy did not change with season or age (Table 2). Sex was a significant factor for failure energy ($p < 0.0001$) and approached significance for ultimate stress ($p = 0.070$), but did not affect modulus of toughness ($p = 0.148$). Failure energy was 76% larger and ultimate stress was 5% larger in male compared to female bears. The 95% confidence interval for statistical power in detecting an 11.8% relative change in ultimate stress ranged from 76% to 97%, with a calculated power of 90%.

Ash fraction

Ash fraction was not different ($p = 0.814$) between fall and spring bears but did increase with age ($p < 0.0001$) (Table 2, Figure 2). Sex was not a significant factor for ash fraction ($p = 0.360$). Statistical power to detect a 25% relative change (between pre- and post-hibernation) in ash fraction was greater than 99%.

Porosity

Four samples (male spring bears: 2, 4, 7, and 15 years old) were lost during processing. Thus $n = 61$ for porosity analyses (22 fall bears, 39 spring bears). Porosity was not different ($p = 0.304$) between fall and spring bears, but interestingly, porosity decreased with age ($p < 0.0001$) (Table 2, Figures 3–4). Sex was not a significant factor for porosity ($p = 0.448$). Statistical power to detect a 333% relative change in porosity was greater than 99%.

Discussion

Prolonged reductions in physical loading of the skeleton (> 1 month) usually lead to cortical bone loss including decreased bone geometrical properties, increased intracortical porosity and decreased bone strength. For example, bone fracture rates are elevated in humans with spinal cord injuries (Vestergaard et al., 1998) because cortical bone area, thickness, and mineral density decline after paralysis (Eser et al., 2004). Similarly, cortical bone area and mineral density are decreased and intracortical porosity is increased in limb-immobilized animals (Lane et al., 1996; Gross et al., 1999; Li et al., 2005) which reduces whole bone strength (Kaneps et al., 1997). In contrast, this study showed that bears prevent cortical bone loss during disuse: cortical bone structure, strength, and mineralization were not different between pre- and post-hibernation bears. This likely occurred because hibernating bears decrease cortical bone remodeling while maintaining a balance between bone formation and bone resorption (McGee et al., 2008).

The bone geometrical and mechanical properties quantified in this study are consistent with previous work on bears. For example, it was previously shown that cortical bone area in the humerus is not different between fall and spring black bears (Pardy et al., 2004) and that bone geometrical properties of the femur are not different between active and hibernating grizzly bears (McGee et al., 2008). These data were supported by the current study in that bone geometrical properties in the femur were not different between fall and spring black bears (Table 2). An advantage of the current study over previous work is the large sample size ($n = 65$ bears) which gave high statistical power for these analyses. Sex differences were noted in the bone geometrical properties as was previously seen in fall black bears from Utah (McGee et al., 2007b). This effect is probably due to differences in body size between male and female bears, since bone cross-sectional properties in the femoral diaphysis are proportional to body weight (Stein et al., 1998), and male bears generally have a greater body mass than female bears (Blanchard, 1987; Parkhurst, 1998; Derocher et al., 2005). Bone properties that may be independent of body mass (e.g., porosity, ash fraction) were not different between male and female bears, which is consistent with the concept that the sex differences observed in this study are probably linked to differences in body size. The age-related trends in bone geometrical and mechanical properties are also comparable to prior work. Bears in this study increased cortical bone area and moments of inertia and decreased modulus of toughness with age (Table 2), similar to trends seen in fall black bears in Michigan and Utah (McGee et al., 2007a; McGee et al., 2007b). The increasing trend for ultimate stress with age approached significance ($p = 0.058$, Table 2), whereas previously we found that ultimate stress significantly increases with age in skeletally immature black bear femurs (McGee et al., 2007a). The lack of significance in the current study is probably due to the influence of a data point from a skeletally mature bear (19 year old female fall bear). Cook's distance for this data point was

0.98, indicating that it likely affected the regression model. Without this data point, ultimate stress significantly increased ($p = 0.0005$, $r = 0.358$) with age.

It was previously shown that intracortical porosity was 30% lower and ash fraction was 2% higher in hibernating compared to active grizzly bears (McGee et al., 2008). In contrast, differences in porosity and ash fraction between fall and spring black bears did not achieve statistical significance (Table 2, Figures 3–4). Disparity in the statistical results between these studies may be because the spring bears in this study experienced some remobilization. Bears resume many physiological processes at the onset of remobilization (e.g., waste excretion); bone turnover is probably increased soon after physical activity is resumed following hibernation (McGee et al., 2008). The radial rate of bone resorption by osteoclasts in cortical bone is approximately $9 \mu\text{m/day}$ (Jaworski et al., 1975), and active bears demonstrate an activation frequency of intracortical remodeling of approximately $0.5 \text{ sites/mm}^2/\text{week}$ (McGee et al., 2008); a bear with a 300 mm^2 femoral cross-sectional area could activate approximately 140 new remodeling sites each week, and each site could become a $100\text{--}200 \mu\text{m}$ diameter remodeling cavity in 1–2 weeks. Therefore, even if porosity decreased in the black bears during hibernation (as occurs in hibernating grizzly bears), it is possible that the spring bears experienced some degree of bone resorption during their 1–4 weeks of remobilization which began to elevate porosity. However, most notably, porosity was not higher in bears following 6 months of disuse, unlike the increased porosity observed in limb immobilized turkeys and dogs (Lanyon and Rubin, 1984; Gross and Rubin, 1995; Li et al., 2005). An increase in bone remodeling immediately following hibernation could also explain why ash fraction was not significantly different between the spring and fall bears, since newly remodeled bone is less mineralized than older bone.

The mechanism by which bears prevent disuse induced bone loss is likely related to calcium recycling. Bone resorption induced by disuse releases calcium into the circulation; this calcium must subsequently be excreted to maintain homeostatic serum calcium levels. For example, human fecal and urinary calcium excretion increased 20–39% during 17 weeks of bedrest relative to baseline measurements (Shackelford et al., 2004). Since bears do not excrete waste during hibernation (Folk, 1967; Nelson et al., 1973) calcium released via bone resorption would quickly build to toxic levels if the calcium was not recycled. Consequently, bears likely have evolved biological mechanisms to recycle calcium to prevent hypercalcaemia during hibernation. Bears maintain homeostatic serum calcium levels throughout the year (Floyd et al., 1990) though they do not eat, drink, or excrete waste during hibernation. Bone formation remains balanced with bone resorption in hibernating bears (McGee et al., 2008), unlike the imbalance in resorption and formation that occurs during disuse in humans and other animals (Weinreb et al., 1989; Turner et al., 1995; Zerwekh et al., 1998; Li et al., 2005). Thus, calcium released by bone resorption in hibernating bears can be recycled back into the skeleton by the preservation of bone formation processes during hibernation. Circulating factors such as norepinephrine and parathyroid hormone (PTH) may help regulate bone remodeling processes and prevent disuse induced bone loss in hibernating bears. Norepinephrine, which acts through the adrenergic receptor $\beta 2\text{AR}$ on osteoblasts and leads to bone resorption (Moore et al., 1993; Eleftheriou et al., 2005), is decreased in hibernating bear serum relative to pre-hibernation levels ($p = 0.006$, unpublished data). Decreased serum norepinephrine could be involved in a centrally regulated (i.e., hypothalamus controlled) mechanism to decrease bone turnover in hibernating bears to promote the conservation of metabolic energy. PTH is the primary regulator of serum calcium levels; intact PTH could promote osteoblast survival under pro-apoptotic disuse conditions and carboxyl-terminal PTH fragments could reduce bone resorption by osteoclasts, thus maintaining balanced bone remodeling during hibernation (Divieti et al., 2002; Bellido et al., 2003; Donahue et al., 2006; Dufour et al., 2007; McGee et al., 2008).

In conclusion, the current study provides strong support for the idea that bears prevent disuse osteoporosis during 6 months of hibernation. Post-hibernation black bears did not demonstrate losses of bone geometrical or mechanical properties, decreased bone mineralization, or increased porosity compared to pre-hibernation bears. This phenomenon may be related to calcium recycling mechanisms employed by bears during hibernation. Further research into neurological and hormonal control of bone remodeling in hibernating bears may lead to novel therapies for treating osteoporosis.

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Figure 1.

Digitized cross-section of a black bear femoral midshaft. The neutral axis for bending, centroid of the cross-section, moments of inertia about the anatomical axes, and x - and y -distances to the point furthest from the neutral axis were computed with a custom macro and were used to calculate whole bone mechanical properties.

Figure 3. Porosity decreased with age ($p < 0.0001$) but was not different ($p = 0.304$) between fall and spring black bears

Figure 4.

Porosity was not different between fall and spring black bears ($p = 0.304$). These representative histological images are from the lateral quadrant of the femur from 3 year old male bears killed in the fall (pre-hibernation) and spring (post-hibernation). 20X original magnification.

Table 1

Disuse-induced changes in bone properties observed in animal models of disuse. Relative percentage change (disuse: control) represents the relative percentage increase or decrease of the bone property in animals subjected to disuse conditions compared to active controls. These percentage changes were used to calculate beta and statistical power for comparisons of pre- and post-hibernation black bear bone properties. See text for explanations of abbreviations.

Property	Disuse Model	Relative Percentage Change (Disuse: Control)	Reference
Ct.Ar, mm ²	Turkeys, 8 weeks limb immobilization	-12.8%	(Bain and Rubin, 1990)
Ct.Th, mm	Rats, 8 weeks neurectomy	-9.4%	(Iwamoto et al., 2005)
σ_U , MPa	Dogs, 16 weeks limb immobilization	-11.8%	(Kaneps et al., 1997)
ash fraction	Dogs, 16 weeks limb immobilization	-25.0%	(Kaneps et al., 1997)
porosity, %	Turkeys, 8 weeks limb immobilization	+333.0%	(Bain and Rubin, 1990)

Means, standard deviations, and statistics for each bone property investigated. "Season p-value" is for the ANCOVA comparison between seasons. "Age p-value" and "Age r" (where "r" is the coefficient of correlation) are statistics describing age-related trends in bone properties. Sex was a significant model term ($p < 0.0007$) for most bone properties. Sex approached significance for σ_U ($p = 0.070$) but was not significant for ash fraction ($p = 0.360$), u ($p = 0.148$) or porosity ($p = 0.448$). See text for explanations of abbreviations.

Table 2

Property	Fall	Spring	Season p-value	Age p-value	Age r
Ps.Ar (mm ²)	471 (113)	474 (106)	0.673	< 0.0001	+0.394
Ct.Ar (mm ²)	313 (77)	312 (76)	0.524	< 0.0001	+0.384
Es.Ar (mm ²)	158 (42)	162 (38)	0.969	0.0002	+0.339
I _{ML} (mm ⁴)	14463 (7123)	13825 (5841)	0.302	< 0.0001	+0.380
I _{AP} (mm ⁴)	19215 (9493)	20092 (9179)	0.924	< 0.0001	+0.413
I _{max} (mm ⁴)	19555 (9496)	20417 (9295)	0.914	< 0.0001	+0.412
SM (mm ³)	1266 (478)	1239 (415)	0.416	< 0.0001	+0.358
Ct.Th (mm)	4.9 (0.6)	4.8 (0.8)	0.434	< 0.0001	+0.333
σ_U (MPa)	238 (42)	233 (27)	0.483	0.058	+0.171
U _f (J)	46 (15)	43 (16)	0.248	0.588	-0.167
u (mJ/mm ³)	6.1 (1.5)	5.5 (1.6)	0.226	< 0.0001	-0.562
ash fraction	0.694 (0.011)	0.696 (0.010)	0.814	< 0.0001	+0.782
porosity (%)	5.5 (2.2)	4.8 (1.6)	0.304	< 0.0001	-0.557