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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

Meghan E. McGee-Lawrence [a], Samantha J. Wojda [a], Lindsay N. Barlow [a], Thomas D. Drummer [b], Kevin Bunnell [c], Janene Auger [d], Hal L. Black [d], Seth W. Donahue [a,*]

[a] *Department of Biomedical Engineering, Michigan Technological University, 309 Minerals and Materials Engineering Building, 1400 Townsend Drive, Houghton, MI 49931, USA*
[b] *Department of Mathematical Sciences, Michigan Technological University, Houghton, MI 49931, USA*
[c] *Utah Division of Wildlife Resources, 1594 W. North Temple, Salt Lake City, UT 84116, USA*
[d] *Department of Integrative Biology, 401 WIDB, Brigham Young University, Provo, UT 84602, USA*

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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: $5.5\pm2.2\%$; spring: $4.8\pm1.6\%$) 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 osteoporoses.

1. 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, b). 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

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* Corresponding author. Tel.: +1 906 487 1729; fax: +1 906 487 1717.
E-mail address: swdonahu@mtu.edu (S.W. Donahue).

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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).

2. Methods

2.1. 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.

2.2. 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.

2.3. 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 (Fig. 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).

2.4. Whole bone mechanical properties

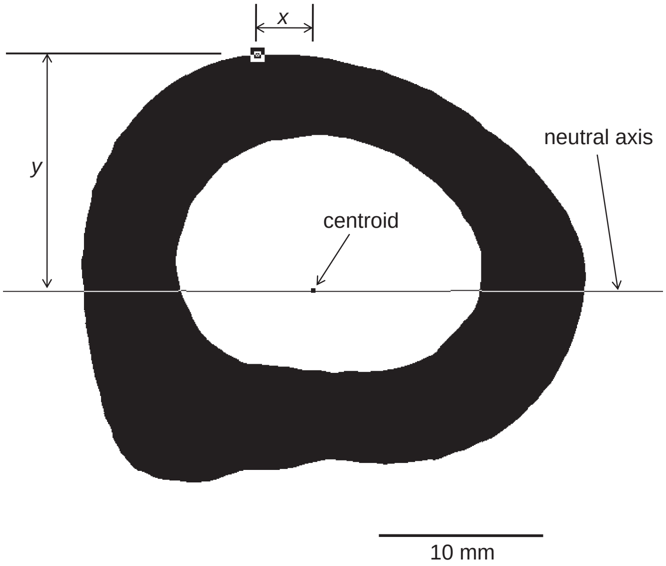

Fig. 1. Digitized cross-section of a black bear femoral midshaft. The neutral axis for bending, centroid of the cross-section, moments of inertia about the anatomical axes, and x- and y-distances to the point furthest from the neutral axis were computed with a custom macro and were used to calculate whole bone mechanical properties.

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

$$\sigma = \frac{PL(I_{AP}y - I_P x)}{4(I_{ML}I_{AP} - I_P^2)} \tag{1}$$

where P was the load and L was the span between the lower supports. Ultimate stress (σ_u) was calculated from Eq. (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 Eq. (2) (Turner and Burr, 2001)

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

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

2.5. 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 h and ashed at 600 °C for 48 h. The ash fraction (a measure of mineral content) was calculated as the ash mass divided by the dry mass.

2.6. 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 s each and rinsed in a 100% ethanol wash for 30 s. The sections were imaged at 40 × 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.

2.7. 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 the 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