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Yellow-bellied Marmots (*Marmota flaviventris*) preserve bone strength and microstructure during hibernation

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ABSTRACT

Reduced skeletal loading typically results in decreased bone strength and increased fracture risk for humans and many other animals. Previous studies have shown bears are able to prevent bone loss during the disuse that occurs during hibernation. Studies with smaller hibernators, which arouse intermittently during hibernation, show that they may lose bone at the microstructural level. These small hibernators, like bats and squirrels, do not utilize intracortical remodeling. However, slightly larger mammals like marmots do. In this study we examined the effects of hibernation on bone structural, mineral, and mechanical properties in yellow-bellied marmots (*Marmota flaviventris*). This was done by comparing cortical bone properties in femurs and trabecular bone properties in tibias from marmots killed before hibernation (fall) and after hibernation (spring). Age data were not available for this study; however, based on femur length the post-hibernation marmots were larger than the pre-hibernation marmots. Thus, cross-sectional properties were normalized by allometric functions of bone length for comparisons between pre- and post-hibernation. Cortical thickness and normalized cortical area were higher in post-hibernation samples; no other normalized cross-sectional properties were different. No cortical bone microstructural loss was evident in osteocyte lacunar measurements, intracortical porosity, or intracortical remodeling cavity density. Osteocyte lacunar area, porosity, and density were surprisingly lower in post-hibernation samples. Trabecular bone volume fraction was not different between pre- and post-hibernation. Measures of both trabecular and cortical bone mineral content were higher in post-hibernation samples. Three-point bending failure load, failure energy, elastic energy, ultimate stress, and yield stress were all higher in post-hibernation samples. These results support the idea that, like bears, marmots are able to prevent disuse osteoporosis during hibernation, thus preventing increased fracture risk and promoting survival of the extreme environmental conditions that occur in hibernation.

Introduction

Disuse osteoporosis, bone loss caused by decreased physiological levels of mechanical loading, is a problem for many people. It affects astronauts exposed to microgravity, patients confined to bed rest for a variety of medical conditions, and patients with spinal cord injury [1]. During periods of reduced skeletal loading, the bones of humans and many other animals undergo a remodeling process which degrades bone (e.g., increased porosity, decreased bone mineralization) [2]. These changes decrease bone strength and increase fracture risk [3,4]. However, this bone loss with periods of disuse is not evident in some hibernating mammals [5].

Hibernating animals are an interesting animal model of disuse because they experience prolonged annual periods of reduced physical activity (as long as ~6–8 months per year). Early studies suggest that similar to humans, small mammalian hibernators (e.g., bats, hamsters) demonstrate an osteoporotic response (at the microstructural level) during disuse (hibernation) [6,7]. However, recent studies in golden mantled ground squirrels and 13-lined ground squirrels have shown small hibernators are able to maintain bone strength during hibernation [8,9]. A limitation of studying small mammalian hibernators is they do not demonstrate intracortical remodeling, an important mechanism for disuse-induced bone loss in larger species [2]. Larger hibernating mammals like bears and marmots do utilize intracortical remodeling. Bears have been shown to preserve bone throughout hibernation by maintaining balanced bone resorption and formation [5,10]. However, the effect of hibernation on the skeletal system of marmots has not been extensively studied. Bone cross-sectional area, density, and biomechanical indices were not different between pre- and post-hibernation

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woodchucks [11]. Woodchuck serum calcium concentration was higher during the summer than in pre- and post-hibernation groups possibly indicating increased bone metabolism during summer months, however serum metabolites during hibernation were not examined [12].

Yellow-bellied marmots (*Marmota flaviventris*) hibernate approximately 6–8 months of the year [13,14]. Throughout this hibernation period, marmots (like bears) do not eat and instead rely on fat stores for metabolic energy [15–17]. Unlike bears however, marmots urinate to excrete waste (which could potentially include calcium and other catabolic bone products) during hibernation [18]. Like other small hibernators marmots experience intermittent bouts of arousal [19,20]. However, unlike other small hibernators yellow-bellied marmots are large enough to utilize intracortical remodeling [21–23]. This study examined the effects of hibernation on cortical and trabecular bone properties in yellow-bellied marmots. We hypothesized that post-hibernation marmots would demonstrate both cortical and trabecular bone loss compared to pre-hibernation marmots because they are able to excrete calcium and other catabolic bone products during interbout arousals during the hibernation period.

Methods

Samples

Sixty-six yellow-bellied marmots (*Marmota flaviventris*) collected in Utah between March 2009 and September 2010 were used for this study. There were 34 pre-hibernation samples (collected between August 5th and September 2nd) and 32 post-hibernation samples (collected from March 22nd through the beginning of June). Dates of capture were within several weeks of immergence or emergence from hibernation. Generally, marmots are expected to enter hibernation by October and emerge in April or May [13,24]. Age data were not available. Sex and weight data were only available for some samples. Hind limb bones were removed post mortem, cleaned of soft tissue and stored at −20 °C.

The right femur was stored at −20 °C until mechanical testing. The distal ½ of the left femur from each marmot was removed and cleaned of marrow. Each segment was fixed in 70% ethanol for a minimum of 48 hours then bulk stained in Villanueva osteochrome bone stain. Stained segments were embedded in methyl-methacrylate (Ortho-jet, Lang Dental Manufacturing Co, Inc) then cut perpendicular to the longitudinal axis, starting at the midshaft, on an Isomet 1000 diamond saw (Buehler LTD). The first section (i.e., the femoral midshaft) was saved for quantification of bone geometrical properties. Subsequent sections were ground to <90 microns and mounted onto microscope slides.

Whole bone bending

The right femur of each animal was thawed and rehydrated in 0.15 M saline solution for approximately 5 hours prior to mechanical testing. Each femur was loaded to failure in three-point bending with the anterior side of the bone in tension. Tests were performed on an Instron mechanical testing system (Instron Model #8872, Canton, MA) with a crosshead speed of 10 mm/min using an adjustable span test fixture with rounded supports ($r = 3.7$ mm). Data was collected at a sampling rate of 1.0 kHz. Due to the size and geometry of the bones a small pre-load (1–10 N) was applied to ensure the bone did not rotate during loading. Average span for testing was 36.2 mm and average span/diameter ratio was 6.3.

Geometrical properties

A cross-section at the femur midshaft of each sample was imaged using a Nikon lens and spot digital camera (SPOT Insight QE, Diagnostic Instruments, Sterling Heights, MI). Periosteal area (Ps.Ar), cortical area (Ct.Ar), and endocortical area (Ec.Ar) for each sample were calculated using image analysis software (Scion Corporation, Frederick, Maryland). A custom macro was utilized with this image analysis software 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 [9]. Cortical thickness (Ct. Th) was calculated in 0.1 mm increments for the cross section using Bioquant Osteo II (Nashville, TN).

Whole bone mechanical properties

Whole bone mechanical properties were calculated using beam bending theory [25]. Stress was determined as follows:

$$\sigma = \frac{P * L * \left(I_{AP*y} - I_{P*x}\right)}{4 * \left(I_{ML} * I_{AP} - I^2{}_P\right)} \quad (1)$$

For this equation P is the load and L is the span between lower supports. Ultimate stress (σ_u) was calculated using Eq. (1), where P is the ultimate load (P_u). Failure energy (U_f) was calculated as the area under the load–deformation curve up to the failure point. Modulus of toughness (u) was calculated using Eq. (2)[26] where c is one-half the anterior–posterior diameter:

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

Elastic energy (U_e) or the energy up to the yield point was also determined. Elastic energy was determined as the area under the load–deformation curve up to the value corresponding to yield stress. Yield stress was determined using the 0.2% offset method. For conversions from load to stress Eq. (1) was used. Deformation was converted to strain using Eq. (3) where d is the deformation [26].

$$\varepsilon = \frac{12 * c * d}{L^2} \quad (3)$$

Resilience was calculated as modulus of toughness to yield (replacing U_f in Eq. (2) with U_e). Elastic modulus was calculated using Eq. (4)[26], where (P/d) is the stiffness (slope of the linear region of the load–deformation curve).

$$E = \left(\frac{P}{d}\right) * \left(\frac{L}{48 * I_{ML}}\right) \quad (4)$$

Ash fraction

To obtain a measure of mineral content, ash fraction for each sample was determined from the cortical bone of the proximal ½ of the femoral diaphysis. Each section was cleaned of marrow with a water jet and rehydrated in 0.15 M saline for 20 minutes. Segments were weighed (wet mass) then placed in a furnace and dried at 100 °C for 24 hours and re-weighed to obtain a dry mass. Samples were placed back in the furnace and ashed at 600 °C for 48 hours. After 48 hours the furnace was turned down to 100 °C, after the samples reached 100 °C they were weighed again (ash mass). The ash fraction was calculated as the ash mass divided by the dry mass.

Histology

Lacunar properties

Osteocyte lacunar properties were quantified for one femoral cross section per marmot. The cross-section was divided into octants and two pictures were taken for each octant, one image of the periosteal