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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.

Keywords

Hibernation; Yellow-bellied marmot; Disuse osteoporosis

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 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 $\frac{1}{2}$ 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:

$$\sigma = \frac{P * L * (I_{APxy} - I_{Pxy})}{4 * (I_{ML} * I_{AP} - I_p^2)} \quad (1)$$

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:

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

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 side and one of the endocortical side. Images were taken at 400× magnification on an Olympus CX41 microscope (Center Valley, PA) and Spot camera (SPOT Insight QE, Diagnostic Instruments, Sterling Heights, MI). Lacunar measurements were made using an image analysis software package, Bioquant Osteo II (Nashville, TN). All lacunae in focus in the field of view for each image were measured. Lacunar properties quantified for this study were average osteocyte lacunar area (Lc.Ar), lacunar density (Lc.Dn), and lacunar porosity (Lc.Por) (total lacunar area divided by bone area).

Resorption and refilling cavities—Remodeling (including both resorption and refilling) cavity density was quantified for three consecutive femoral cross-sections starting at the midshaft. Resorption and refilling cavity density was calculated as the number of resorption or refilling cavities per cross-section area.

Intracortical porosity—Intracortical porosity was quantified for one femoral cross section per marmot. The cross section for each sample was imaged at 100× magnification using an Olympus CX41 microscope (Center Valley, PA) and Spot camera (SPOT Insight QE, Diagnostic Instruments, Sterling Heights, MI). Images were analyzed in Bioquant Osteo (Nashville, TN). Porosity was calculated as porous area divided by total bone area. Porous area included Haversian canals and Volkmanns canals as well as any resorption or refilling cavities, but not osteocyte lacunae.

Micro computed tomography (μ CT)—The left tibia from each sample was fixed in 70% ethanol. Trabecular bone properties were assessed by micro computed tomography (μ CT). The proximal tibia was scanned at 30 μ m resolution (μ CT 35, Scanco Medical). During scanning bones were held in a 30.5 mm tube surrounded by ethanol. To ensure that the same relative location was scanned in bones of different sizes, the scanned and evaluated regions for each bone were scaled to bone length (Fig. 1). Scans started at a distance of 3–9.96 mm distal to the proximal tibial growth plate (the starting point for scans was equal to a distance of 6% of the total bone length below the growth plate, and the length of the evaluated region was equal to 5% of the total bone length).

Statistics

Because post-hibernation animals were larger than pre-hibernation animals, cross-sectional properties were normalized by functions of femur length then compared between pre- and post-hibernation groups with a *t*-test. Femur length was chosen for normalization because it is known that cross-sectional area scales approximately with (femur length)² and moments of inertia scale with (femur length)⁴ in various species [27,28].

Mechanical properties, ash fraction, remodeling cavity density, intracortical porosity, and trabecular μ CT data were compared between groups with *t*-tests. In order to more clearly examine seasonal changes in these properties, without animal size effect, paired *t*-tests were performed. To do this samples were paired based on femur length ($n=11$ per season).

Osteocyte lacunar data were analyzed using a three-way ANOVA with season, octant, and cortical location (endocortical or periosteal) as factors. Similar to the other properties, paired *t*-tests were used to determine whether or not properties were different between pre- and post-hibernation samples reducing the effect of size as a confounding variable.

Sex and weight were not included in any analyses because these data were not available for all samples. However, within the samples where sex data were available the distribution of males and females was similar between pre- and post-hibernation groups.

Results

Samples

Since age data were not available for any samples and bodyweight data were only available for a subset of samples, femur length was used to determine if groups contained animals of similar size. The spring group femurs were 12.5% longer than those in the pre-hibernation group ($p<0.0001$). Similarly, for those samples where weight data were available, femurs were 14.5% longer ($p<0.0001$), and weight was 23% higher ($p=0.0285$) in the post-hibernation samples. Marmots normally lose $32.5\pm 2.5\%$ of their pre-hibernation body weight during hibernation [29,30]. Thus, it is reasonable to conclude post-hibernation animals were larger and probably older than pre-hibernation samples in this study. When possible, and appropriate, data in this study were adjusted or paired to account for these differences in size. However, it is important to note that these adjustments cannot entirely account for differences in size between groups.

Geometrical properties

Cortical thickness ($p<0.0001$) and normalized cortical area ($p=0.001$) were unexpectedly higher post-hibernation as compared to pre-hibernation animals (Table 1). Size adjusted endocortical area was lower ($p=0.0003$) in post-hibernation samples. Normalized periosteal area was not different ($p=0.1374$) between groups. Normalized moments of inertia were not

different between groups, indicating resistance to bending is preserved during hibernation. Together these data indicate cross-sectional properties are preserved during hibernation.

Whole bone bending

Some samples had to be excluded from the mechanical property calculations due to missing cross-sectional data or lack of an obvious failure point on the stress–strain curve. Sample sizes were 21 for pre-hibernation and 27 for post-hibernation for failure load, displacement, and energy. Modulus of toughness sample size was 19 for pre-hibernation and 27 for post-hibernation. Ultimate stress sample size was 29 for pre-hibernation and 30 for post-hibernation.

Failure load, failure energy, elastic energy, ultimate stress, and yield stress were all higher ($p<0.004$) in post-hibernation as compared to pre-hibernation samples (Table 2). Modulus of toughness was lower ($p=0.0431$) in post-hibernation samples. There were no differences ($p>0.319$) in resilience, elastic modulus, or failure displacement between pre- and post-hibernation samples. When samples were paired by femur length (to account for differences in bone size) there were no differences between groups for any of the mechanical properties (Table 3). Thus, it appears as though marmots maintain their ability to resist fracture within normal physiological loading even after the disuse associated with hibernation.

Ash fraction

Ash fraction was 3.3% higher in post-hibernation samples ($p=0.0003$). The apparent increase in mineralization in the post-hibernation group is likely an effect of having larger/older animals in that group. An increase in ash fraction with age has been observed in bears and 13-lined ground squirrels [5,9]. The increase in ash fraction may also explain the decrease in modulus of toughness with hibernation in unpaired data. When data were paired by femur length there was no difference ($p=0.387$) in ash fraction between pre- and post-hibernation samples. Taken together these findings indicate that cortical bone mineral content is not adversely affected by hibernation.

Histology

Lacunar properties—Osteocyte lacunar area was 9.3% lower in post-hibernation samples ($p<0.0001$) compared to pre-hibernation samples (Table 4). Average lacunar area was larger in the periosteal regions ($p<0.001$) compared to endocortical regions and varied depending on which octant the lacunae were from ($p<0.0001$). Lacunar density was 10.3% lower in the post-hibernation group ($p<0.0001$). Lacunar porosity was 21% lower in the post-hibernation group ($p<0.0001$). Lacunar porosity varied by octant ($p=0.0162$) but not by endocortical/periosteal region ($p=0.3418$). Lacunar density was lower in the periosteal regions ($p<0.0001$) and varied by octant ($p<0.0001$). Though there was some variation of lacunar properties within a cross-section, it is most important to note that lacunar area, density, and porosity were all lower ($p<0.0001$) in post-hibernation samples. Paired analysis also showed osteocyte lacunar area ($p=0.0164$), lacunar porosity ($p=0.0067$) and lacunar density tended ($p=0.0606$) to be lower in post-hibernation samples (Table 5). These findings are indicative of preservation of bone, as lacunar area and porosity would be expected to be higher in post-hibernation samples if bone were being lost through osteocytic osteolysis, as in previous reports on hibernating mammals [6,31].

Resorption and refilling cavities—Both resorption and refilling cavity density were not different between pre- and post-hibernation samples ($p>0.37$ for both). Remodeling cavities were not present in all samples. However, at least one remodeling cavity was present in 25/34 pre-hibernation samples and 26/32 post-hibernation samples.

Intracortical porosity—Intracortical porosity was not different (ANOVA, $p=0.6336$; paired t -test, $p=0.9775$) between pre- and post-hibernation samples. Periosteal bone formation was observed, as a relatively large number of primary osteons forming on the periosteal surface, in 14 of the 31 pre-hibernation samples. The samples showing periosteal bone formation were spread throughout the size range of femurs and the collection period, there were no obvious connections to either size or collection period. During growth, bone formation occurs on the periosteal surface, increasing the bone diameter. As this occurs blood vessel canals are occasionally open to the periosteal surface [32]. This was only noticed in 3 of the 31 post-hibernation samples; however these 3 samples were collected the furthest into the active season. This may suggest growth is greatly reduced, if not suspended, during hibernation. This increased periosteal formation was not taken into account in quantification of refilling cavities because they were not secondary remodeling cavities.

Micro computed tomography (μ CT)—Micro-computed tomography of the proximal tibia showed trabecular bone volume fraction in the proximal tibia tended to be higher in post-hibernation samples ($p=0.0548$). Trabecular number ($p=0.0187$) was 16.6% lower in post-hibernation samples (Table 6). Trabecular separation was 14.6% higher post-hibernation ($p=0.0396$). Trabecular thickness was not different between groups ($p=0.8233$). Material mineral density was 4.2% higher in the post-hibernation samples ($p<0.0001$). Paired t -tests showed no differences between groups for bone volume fraction, trabecular number, trabecular thickness, trabecular separation, or material mineral density ($p>0.326$). Based on these data, trabecular bone architectural properties are preserved during hibernation (Fig. 2).

Discussion

Hibernating mammals have a unique ability to reduce their energy expenditure in order to adapt to stressful conditions, like the cold temperatures and food scarcity of winter [19]. Animals that hibernate for the winter are inactive for up to 6–8 months of the year [13,14,33]. If humans and many mammals were to remain inactive or significantly decrease skeletal loading for a similar period of time, the musculo-skeletal system would be impaired [1,34]. Bears have been shown to preserve bone integrity during the inactive period of hibernation [5,10]. Understanding the mechanisms that preserve bone in hibernators could lead to translating the mechanism to a treatment for disuse osteoporosis in humans. Smaller hibernators may be a more practical model for mechanistic studies, as we found marmots and previous studies showed squirrels [8,9] do not lose bone during hibernation. An advantage of marmots over other small hibernators is they are large enough to utilize intracortical remodeling and have been used in many laboratories for hibernation physiology research [30,35,36].

The present study indicates preservation of bone during hibernation in yellow-bellied marmots. The pre-hibernation group in this study provides a baseline for bone properties in an active marmot prior to entering hibernation. The post-hibernation group was captured within a few weeks of emergence from hibernation. Though some remobilization had occurred, based on the time required for recovery of bone lost during disuse [34], this would not have been sufficient time to regain lost bone if marmot bones were remodeled like those of non-hibernating mammals during disuse. Mechanical and cross-sectional properties were not diminished in bones collected after hibernation. The one exception was the decrease in modulus of toughness in post-hibernation samples for the entire data set. Since larger animals in the post-hibernation group indicate an older group [37], and ash fraction was higher in the post-hibernation group, the decrease in toughness is likely due to increasing mineral with age. Similarly, ultimate stress and ash fraction have been found to increase with age in black bears and 13-lined ground squirrels [5,9]. Because age data were not

available for this study, correlations between mechanical properties and age could not be made. It is important, however, to note that when paired by femur length no differences were observed between pre- and post-hibernation samples for any mechanical property. Similarly, there was no difference in ash fraction between groups when samples were paired by femur length. It is also important to note that yield stress was higher in post-hibernation samples and resilience was not different between groups. This suggests there is no increase in fracture risk with resumption of normal physiological loading in post-hibernation marmots. Thus, the preservation of bone during hibernation contributes to the ability of marmots to survive prolonged extreme environmental conditions.

Micro-structural changes, or lack thereof, also indicate preservation of bone during the hibernation period. There were no differences between groups, in the full data set or paired data, between remodeling cavity density or intracortical porosity. If microstructural bone loss were occurring, it is likely an increased porosity would be observed after hibernation as seen in other disuse models [2,38]. The lack of change in intracortical porosity between pre- and post-hibernation marmots is similar to findings in black bears; porosity was not different between pre- and post-hibernation black bears [5]. However, porosity was 30% lower in hibernating compared to active grizzly bears [39]. Osteocyte lacunar properties were lower in post-hibernation marmots, and thus did not demonstrate the increase in lacunar size that was previously reported to occur in smaller hibernating mammals [6,7]. Similarly, when data were paired based on femur length, lacunar area and lacunar porosity were lower in post-hibernation samples. It is noteworthy that the results of the paired data are comparable to the results from the entire data set because lacunar density has been shown to decrease with age in humans [40]. The decreasing trend in properties after hibernation is also similar to findings in bears. Lacunar porosity was 26% lower in hibernating bears compared to active bears and lacunar size was 15% lower in hibernating bears than in active bears (density was not reported) [41]. Average lacunar area did not change in active vs. hibernating 13-lined ground squirrels. However, lacunar porosity was 44% higher and density was 59% higher in hibernating squirrels [9]. The differences between what was observed in lacunar properties in 13-lined ground squirrels compared to bears and marmots may be a result of how bone is remodeled. Marmots and bears can utilize intracortical remodeling as well as utilizing trabecular bone remodeling and the lacunar–canalicular system to maintain mineral homeostasis during hibernation. Although squirrels can utilize trabecular bone remodeling and osteocytic osteolysis, they are too small for intracortical remodeling. Thus, is it possible that to adequately maintain mineral homeostasis, more mineral needs to be removed via osteocytic osteolysis in squirrels than in marmots or bears, resulting in a measureable increase in lacunar porosity with hibernation in squirrels. The opposite appears to be occurring in marmots and bears. Osteocyte lacunar area and porosity decrease after hibernation, indicating osteocytes may actually be filling in the lacunae. Osteocytic bone formation has been demonstrated by perilacunar tetracycline labeling as well as a measureable decrease in lacunar area [42,43]. This excess mineral may be deposited in the lacunar–canalicular system as a result of hormonal changes and/or suspended periosteal growth during hibernation.

It also appears as though trabecular bone architecture is preserved in hibernating marmots. However, trabecular number was lower and trabecular separation was higher in post-hibernation samples when the entire data set was analyzed, and material mineral density was higher in post-hibernation samples. Paired *t*-tests showed no differences in any trabecular bone architectural properties between groups. This further indicates prevention of bone loss during hibernation in marmots. Trabecular bone properties have also been shown to be preserved in hibernating bears and juvenile 13-lined ground squirrels [10,44].

Overall, the results of this study indicate preservation of bone mechanical, geometrical, mineral, and microstructural properties in marmots during hibernation. However, the lack of age data is a limitation. Future studies should include age data, which would allow for better comparisons between groups. Though it is not likely much, if any, growth occurs during the hibernation period, it is possible differences between juveniles and adults could affect the analyses. Further characterization of circannual skeletal and hormonal changes in marmots and other hibernators may lead to understanding the mechanism that allows hibernators preserve bone during hibernation. Different species of hibernators may utilize the same or similar physiological mechanism to preserve bone during hibernation. More in depth studies looking at bone cells, endocrine changes, and comparisons across species may allow further understanding of this mechanism. For example, sampling at multiple time-points throughout hibernation and the active seasons would allow characterization of hormonal fluctuations as well as cellular activity and remodeling in bone throughout the year. Preliminary analyses of indicators of bone metabolism in blood serum (i.e., osteocalcin, calcitonin, and leptin) have been made in woodchucks, however, only during the active period [12]. Though these data provide evidence of increased bone metabolism in summer months it does not allow any insight into possible mechanisms for bone preservation during hibernation. Ultimately, understanding skeletal and endocrine changes in hibernation could help lead to an improved treatment for disuse osteoporosis in humans.

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Fig. 1.
Scout view of a marmot tibia with the evaluation region outlined.

Fig. 2.
uCT images of pre- and post-hibernation marmot proximal tibias.

Table 1

Cortical thickness and normalized geometrical properties with % differences in post-hibernation samples relative to pre-hibernation samples.

Property	Pre-hibernation	Post-hibernation	<i>p</i> -Value	% Difference
Cortical thickness (μm)	803 (162)	1120 (291)	<0.0001	28.3
Cortical area	0.00299 (0.00042)	0.00341 (0.000546)	0.0011	12.3
Endocortical area	0.003194 (0.0008)	0.002462 (0.0007)	0.0003	-29.7
Periosteal area	0.0062 (0.001)	0.0059 (0.0006)	0.1374	
I_{max}	2.57 E-6 (7.21 E-7)	2.62 E-6 (5.25 E-7)	0.6330	
I_{AP}	2.56 E-6 (7.15 E-7)	2.62 E-6 (3.34 E-7)	0.6941	
I_{ML}	1.95E-6 (5.5 E-7)	1.92E-6 (3.97 E-7)	0.8090	

Mean values with standard deviations in parentheses.

Significance is highlighted in bold.

Note: Area and moment of inertia measures are size-adjusted values (each measure was divided by the appropriate functions of bone length to provide size-adjusted estimates in order to account for differences in bone size between groups, thus the values in the table are unitless).

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Table 2

Mechanical properties calculated from three-point bending test with % differences in post-hibernation samples relative to pre-hibernation samples.

Property	Units	Pre-hibernation	Post-hibernation	p-Value	% Difference
Failure load	N	214 (91)	403 (150)	<0.0001	46.9
Failure displacement	mm	2.27 (0.81)	2.07 (0.58)	0.319	
Failure energy	mJ	458 (239)	673 (255)	0.0046	31.9
Elastic energy	mJ	62 (41)	105 (46)	0.0007	40.6
Modulus of toughness	mJ/mm ³	9.92 (3.52)	8.12 (2.35)	0.0431	-22.2
Resilience	mJ/mm ³	1.3 (0.68)	1.4 (0.37)	0.5272	
Ultimate stress	MPa	160 (48)	198 (33)	0.0009	19.2
Yield stress	MPa	108 (45)	143 (28)	0.0015	24.3
Elastic modulus	GPa	6.2 (1.9)	6.5 (1.9)	0.4947	

Mean values with standard deviations in parentheses.

Significance is highlighted in bold.

Table 3

There were no significant differences in mechanical properties for paired samples. Twelve samples from each group were paired by femur length.

Property	Units	Pre-hibernation	Post-hibernation	<i>p</i> -Value
Failure load	N	263 (106)	313 (106)	0.2824
Failure displacement	mm	2.36 (1.06)	2.37 (0.95)	0.987
Failure energy	mJ	612 (256)	611 (230)	0.9946
Elastic energy	mJ	78 (41)	81 (39)	0.7846
Modulus of toughness	mJ/mm ³	10.07 (3.67)	8.2 (2.25)	0.2876
Resilience	mJ/mm ³	1.49 (0.69)	1.37 (0.41)	0.5912
Ultimate stress	MPa	176 (49)	187 (39)	0.5033
Yield stress	MPa	123 (46)	134 (33)	0.5048
Elastic modulus	GPa	6.3 (1.7)	6.6 (1.8)	0.7001

Mean values with standard deviations in parentheses.

Significance is highlighted in bold.

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Table 4
Osteocyte lacunar properties with % differences in post-hibernation samples relative to pre-hibernation samples.

Property	Units	Pre-hibernation	Post-hibernation	p-Value	% difference
Average lacunar area	μm ²	49.26 (7.8)	45.08 (7.8)	< 0.0001	-9.3
Lacunar porosity	%	0.95 (0.3913)	0.79 (0.26)	< 0.0001	-21
Lacunar density	#/mm ²	193 (60.9)	175 (30.8)	< 0.0001	-10.3

Mean values with standard deviations in parentheses.

Significance is highlighted in bold.

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Table 5

Data from paired *t*-test (*n*=11 per group) of osteocyte lacunar properties with % differences in post-hibernation samples relative to pre-hibernation samples.

Property	Units	Pre-hibernation	Post-hibernation	<i>p</i> -Value	% Difference
Average lacunar area	μm ²	48.7±5.6	44.6±4.4	0.0164	-9.3
Lacunar porosity	%	0.975±.22	0.78±0.13	0.0067	-24.4
Lacunar density	#/mm ²	0.0002±0.000038	0.000176±0.000025	0.0606	

Mean values with standard deviations in parentheses.

Significance is highlighted in bold.

Table 6

Results of one-way ANOVA comparing pre-hibernation vs. post-hibernation samples for all properties measured by μ CT with % differences in post-hibernation samples relative to pre-hibernation samples.

Property	Units	Pre-hibernation	Post-hibernation	p-Value	% Difference
BV	mm ³	10.4±11.9	10.97±5.1	0.8191	
TV	mm ³	88.6±20.5	94.2±31.9	0.386	
BV/TV	%	9.6±4.5	12.2±6.2	0.0548	
Tb.N	mm ⁻¹	2.74±0.66	2.35±0.65	0.0187	-16.6
Tb.Th	mm	0.094 ±0.1	0.090±0.014	0.8233	
Tb.Sp	mm	0.39 ±0.124	0.458±0.135	0.0396	14.6
App.M.Dn	mgHA/ccm	198±49	200±58	0.8768	
Mat.M.Dn	mgHA/ccm	813±32	849±35	<0.0001	4.2

Mean values with standard deviations in parentheses.

Significance is highlighted in bold.

Abbreviations are as follows; bone volume (BV), tissue volume (TV), bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th.), trabecular separation (Tb.Sp.), apparent mineral density (App.M.Dn), material mineral density (Mat.M.Dn).