Hibernating Little Pocket Mice Show Few Seasonal Changes in Bone Properties

NOELLYN PINEDA,[1,2] MARJORIE OWEN,[2] CLAIRE TUCKER,[2] SAMANTHA WOJDA,[2] STANLEY KITCHEN,[3] HAL BLACK,[4] AND SETH DONAHUE [1,2]*

[1]School of Biomedical Engineering, Colorado State University, Fort Collins, Colorado
[2]Department of Mechanical Engineering, Colorado State University, Fort Collins, Colorado
[3]US Forest Service, Rocky Mountain Research Station, Provo, Utah
[4]Department of Plant and Wildlife Sciences, Brigham Young University, Provo, Utah

ABSTRACT

Periods of disuse or physical inactivity increases bone porosity and decreases bone mineral density, resulting in a loss of bone mechanical competence in many animals. Although large hibernators like bears and marmots prevent bone loss during hibernation, despite long periods of physical inactivity, some small hibernators do lose bone during hibernation. Little pocket mice (*Perognathus longimembris*) remain underground during winter hibernation and undergo bouts of torpor and interbout arousals, but the torpor bout duration is shorter than other rodent hibernators. Additionally, little pocket mice may enter torpor during summer estivation. In this study, cortical and trabecular bone architectural, mineral, and mechanical properties were analyzed for femurs from little pocket mice captured during 8 different months (March to October) to determine seasonal effects on bone. There were no differences in any bone properties between the pre-hibernation month of October and the post-hibernation month of March, suggesting winter hibernation did not adversely affect bone properties. However, cortical area was higher in March than April, May, and June. Bone mechanical and osteocyte lacunar properties were not different between any months. Trabecular bone in the distal femoral epiphysis showed no changes between months. The distal femoral metaphyseal region showed higher trabecular spacing and lower trabecular number in May than August, otherwise, there were no differences in trabecular parameters. The few monthly differences in bone properties may be due to physical inactivity from periodic summer estivation or from the timing of birth and growth in spring and summer months. Anat Rec, 300:2175–2183, 2017.

Key words: bone; hibernation; little pocket mice

During periods of absent or reduced skeletal loading, there is an increased rate of osteoclast-mediated bone resorption and/or inhibition of osteoblast-mediated bone formation (Weinreb et al., 1989; Kaneps et al., 1997). This leads to increased porosity and decreased bone mineral density (BMD) (Noble and Reeve, 2000; Takata and Yasui, 2001). Bone loss resulting from a decrease in mechanical loading is a problem for patients subject to prolonged bed rest, those with localized immobilization caused by spinal cord injuries (Modlesky et al., 2005) or other medical traumas (Soderpalm et al., 2007; McGee et al., 2008), and astronauts exposed to microgravity (Vico et al., 2000; Takata and Yasui, 2001) because bone mechanical properties are decreased and fracture incidence increases

*Correspondence to: Seth Donahue, 300 West Drake Rd, Fort Collins, CO, 80526 E-mail: seth.donahue@colostate.edu

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(Modlesky et al., 2005; Soderpalm et al., 2007). Many animal models of disuse have been utilized to understand the effects of prolonged inactivity on bone morphology, including dogs (Jaworski et al., 1980; Kaneps et al., 1997; Li et al., 2005), mice (Simske et al., 1991; Simske et al., 1992; Sakai and Nakamura, 2001) and hibernating mammals (Whalen et al., 1972; Zimmerman et al., 1976; Steinberg et al., 1981; McGee et al., 2008; McGee-Lawrence et al., 2009; Utz et al., 2009; McGee-Lawrence et al., 2011; Doherty et al., 2012; Wojda et al., 2012; McGee-Lawrence et al., 2015; Doherty et al., 2016; Wojda et al., 2016). Studies on dog limb immobilization demonstrated a decrease in mechanical strength (Kaneps et al., 1997; Li et al., 2005) and macrostructural bone loss (Jaworski et al., 1980; Li et al., 2005). Studies on immobilized mice found similar results with an increased rate of bone resorption associated with microstructural (Sakai and Nakamura, 2001) and macrostructural (Simske et al., 1992) bone loss and minimal effects on bone strength (Simske et al., 1991; Simske et al., 1992). Hibernating mammals have also been used to study the effects of disuse on bone. Several species of bears and rodents drastically reduce metabolism during hibernation to conserve energy when food is scarce (Carey et al., 2003; Toien et al., 2011). Because production of muscle force is metabolically expensive, physical inactivity is substantially decreased during hibernation. Interestingly, studies on bears, marmots and squirrels indicate bone strength and macrostructural properties are preserved despite the disuse associated with hibernation (McGee-Lawrence et al., 2008, 2009, 2011; McGee et al., 2008; Utz et al., 2009; Doherty et al., 2012; Wojda et al., 2012). However, some small hibernating species like bats and hamsters have demonstrated bone loss during hibernation (Whalen et al., 1972; Haller and Zimny, 1977; Steinberg et al., 1981; Kwiecinski et al., 1987; McGee-Lawrence et al., 2008). Many unique bone physiological adaptions have evolved to promote organismal survival in diverse and extreme environments (Doherty et al., 2015). For example, bone remodeling is suppressed, and bone resorption and formation are balanced, in hibernating bears (McGee et al., 2008; McGee-Lawrence et al., 2009, 2015) and rodents (Doherty et al., 2012, 2016; Wojda et al., 2012) despite physical inactivity. Understanding the effects of hibernation on bone in different species of mammalian hibernators, with different physiological changes during hibernation, may lead to insights as to how bone is preserved during hibernation and lead to improved treatments for disuse osteoporosis and other metabolic bone disorders.

Hibernating mammals range in size from small rodents (pocket mice) to grizzly bears. Hibernating rodents and bears have some very different physiological changes during hibernation. For example, during hibernation bears do not experience intermittent bouts of arousal and large body temperature fluctuations, nor do they eat, drink or excrete waste (Nelson, 1973, 1987; Toien et al., 2011b). Small mammalian hibernators on the other hand arouse, for brief periods of time, intermittently from torpor bouts throughout the hibernation season, have large body temperature fluctuations, and may excrete waste and access cached food (Carey et al., 2003; Bartholomew and Cade, 1957). Torpor refers to periods of suppressed metabolism. The hallmark of torpor in rodent hibernators is suppressed body temperature to near ambient temperature. The long winter hibernation season in rodents, like little pocket mice, is characterized by cycles or torpor bouts interrupted by brief interbout arousals which last about 12–24 hr. During interbout arousal, body temperature rises to near normal summer levels, but the animals spend most of the arousal period sleeping and do not leave the hibernaculum. Bears display decreased but balanced bone resorption and formation during hibernation relative to their active state, thus maintaining their bone structure and strength throughout the 5–7 months of physical inactivity due to hibernation (McGee-Lawrence et al., 2008; McGee et al., 2008). Studies on smaller mammalian hibernators show variable results regarding bone metabolism and structure. Studies in little brown bats (*Myotis lucifugus*) and golden hamsters (*Mesocricetus auratus*) indicated possible bone loss during hibernation (Whalen et al., 1972; Steinberg et al., 1981). A more recent study showed juvenile 13-lined ground squirrels preserve cortical bone macrostructure and strength, but some microstructural bone loss was observed (McGee-Lawrence et al., 2011). Whether this bone loss is due to the potential to excrete calcium during the hibernation period or a need to liberate calcium from bone for other biological functions is unclear. Other recent studies have shown that rodent hibernators, such as marmots (Doherty et al., 2012, 2016; Wojda et al., 2012), golden-mantled ground squirrels (Utz et al., 2009), arctic ground squirrels (Wojda et al., 2016), and 13-lined ground squirrels (McGee-Lawrence et al., 2011), are able to maintain bone strength during hibernation. Further investigation into understanding the impact of hibernation on the skeletal system of smaller mammalian hibernators, which arouse intermittently, may help in understanding the skeletal maintenance mechanisms behind bone preservation during hibernation.

This study evaluated the micro- and macro-structural bone properties of the little pocket mouse (*Perognathus longimembris)* throughout spring, summer, and fall. Little pocket mice are the smallest hibernating rodent in North American (Bartholomew and Cade, 1957). They are tiny creatures that live up to their name with a body mass of 6.5–10.5 g and average length of 131 mm, which is less than half the size of the common lab mouse. Little pocket mice are primarily found in desert or grassland regions of Mexico and the southwestern region of the United States. They are nocturnal and spend most of their lives underground; in fact they may spend <1 hr above ground at night (Kenagy, 1973). In the winter, little pocket mice can remain underground for up to 5 months due to the accumulation of food in the hibernaculum during the fall months (Kenagy, 1973). They can also become torpid during summer estivation (Bartholomew and Cade, 1957). Unlike other hibernators, fat accumulation is not a prerequisite for hibernation in pocket mice since food is stored year round and continuous torpor bouts longer than 1 week are uncommon (Bartholomew and Cade, 1957). In a laboratory setting, little pocket mice most commonly underwent sessions of torpor lasting 3 days (Kenagy, 1973). Though rare, between torpor bouts they are capable of returning to the surface to collect seeds for food reserves, but they are not active on the surface below temperatures of $-10°C$ (Kenagy, 1973). The objective of this study was to characterize bone micro- and macro-structural properties throughout the nonhibernation months and consider the

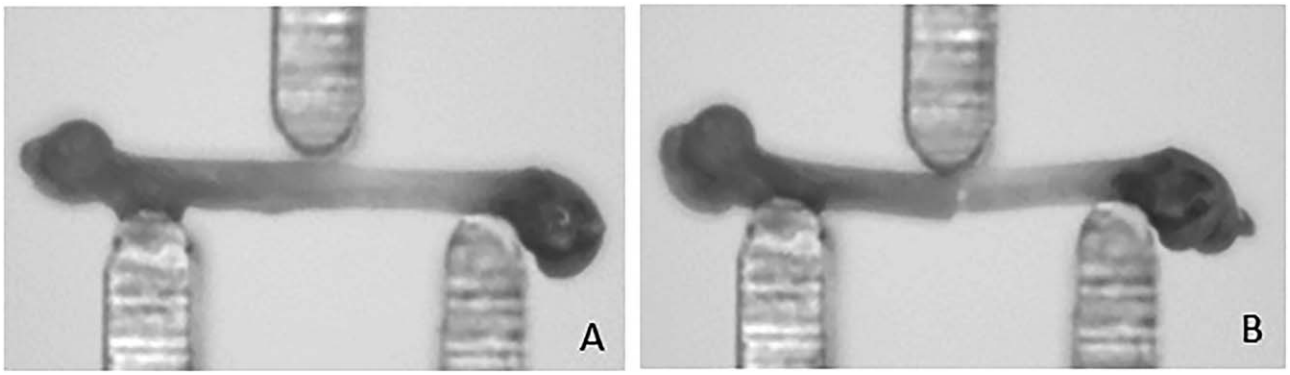

Fig. 1. Left femur of a little pocket mouse on a three-point bend fixture (A) prior to loading and (B) after fracture.

unique physiological characteristics of little pocket mice, to provide additional information for understanding skeletal maintenance during hibernation.

MATERIALS AND METHODS

Samples

One hundred and eighty-seven pocket mice were collected near Milford, Utah (elevation ~1,594 m) between April 2011 and March 2012. Samples were collected throughout 8 months of the year. No animals were collected during winter (November through February). Pocket mice were caught with Victor kill traps using peanut butter and rolled oats for bait in compliance with the Guidelines of the American Society of Mammalogists for the Use of Wild Mammals in Research (Sikes et al., 2011). The specimens were collected with permission from the Utah Division of Wildlife Resources, Central Region, Cedar City UT. The USDA Forest Service Shrub Sciences Laboratory (Provo, UT) permitted collection at the Great Basin Desert Range Experimental Station. The femurs were extracted and stored at −20°C (Martin and Sharkey, 2001). The analyses of the femurs at Colorado State University were exempt from IACUC oversight and the voucher specimens were donated to the Brigham Young University Monte L. Bean Life Science Museum where they are preserved as fluid specimens available for future morphological and genetic research.

The right femoral diaphysis, with marrow removed, was fixed in 70% ethanol for a minimum of 48 hr. After fixation, right femurs were embedded in methylmethacrylate (Ortho-jet, Lang Dental Manufacturing, Wheeling, IL) and sectioned perpendicular to the longitudinal axis at the mid-shaft, on an IsoMet 1000 diamond saw (Buehler, Lake Bluff, IL), ground to ≤90 microns thickness, then mounted on microscope slides.

Whole Bone Bending

The left femur of each sample was thawed and rehydrated in 0.15 M saline solution prior to testing. Femurs were loaded to failure in three-point bending with the posterior side of the bone in tension (Fig. 1). Femurs were positioned on a test fixture with rounded supports (r = 1.5 mm) and a fixed span of 5 mm. Tests were performed on an MTS (Eden Prairie, MN) with a 10 lb LSB303 Futek load cell (Futek Advanced Sensor Technology, Irvine, CA). Tests were run with a crosshead speed of 1 mm min^{-1} and force and displacement data were collected at a sampling rate of 100 Hz.

Femoral Geometrical Properties

An unstained 90-μm thick cross-section at the femur midshaft of each sample was imaged at 40X magnification using a Nikon Eclipse 80i microscope and Olympus DP71 camera. Cortical area (Ct.Ar) for each sample was calculated using Scion Image (Scion, Frederick, ME). A custom macro was used to calculate the cross-sectional moments of inertia about the mediolateral axis (I_{ML}) and the antero-posterior axis (I_{AP}), product of inertia (I_P), maximum moment of inertia (I_{max}), centroid of the cross-section, and neutral axis using Scion Image (Scion, Frederick, ME). Cortical thickness (Ct.Th) was measured in 0.1-mm increments around the entire cross section using Bioquant Osteo (Nashville, TN) then averaged.

Femoral Mechanical Properties

Whole bone mechanical properties were calculated using beam bending theory (Levenston, 1995) and the force-displacement data from three-point bending tests and the cross-sectional geometrical properties. Each bone was considered as an asymmetrical beam. Stress (σ) was calculated using equation (1), where P represents the applied load, x and y represent the distances to the point furthest from the neutral axis and L represents the span between the two lower supports of the test fixture. Ultimate stress was calculated using equation (1):

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

Modulus of toughness (u) was calculated using equation (2) where c represents one-half the anterior–posterior diameter at midshaft:

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

Failure energy (U_f) was determined as the area underneath the load-deformation curve up to the point of

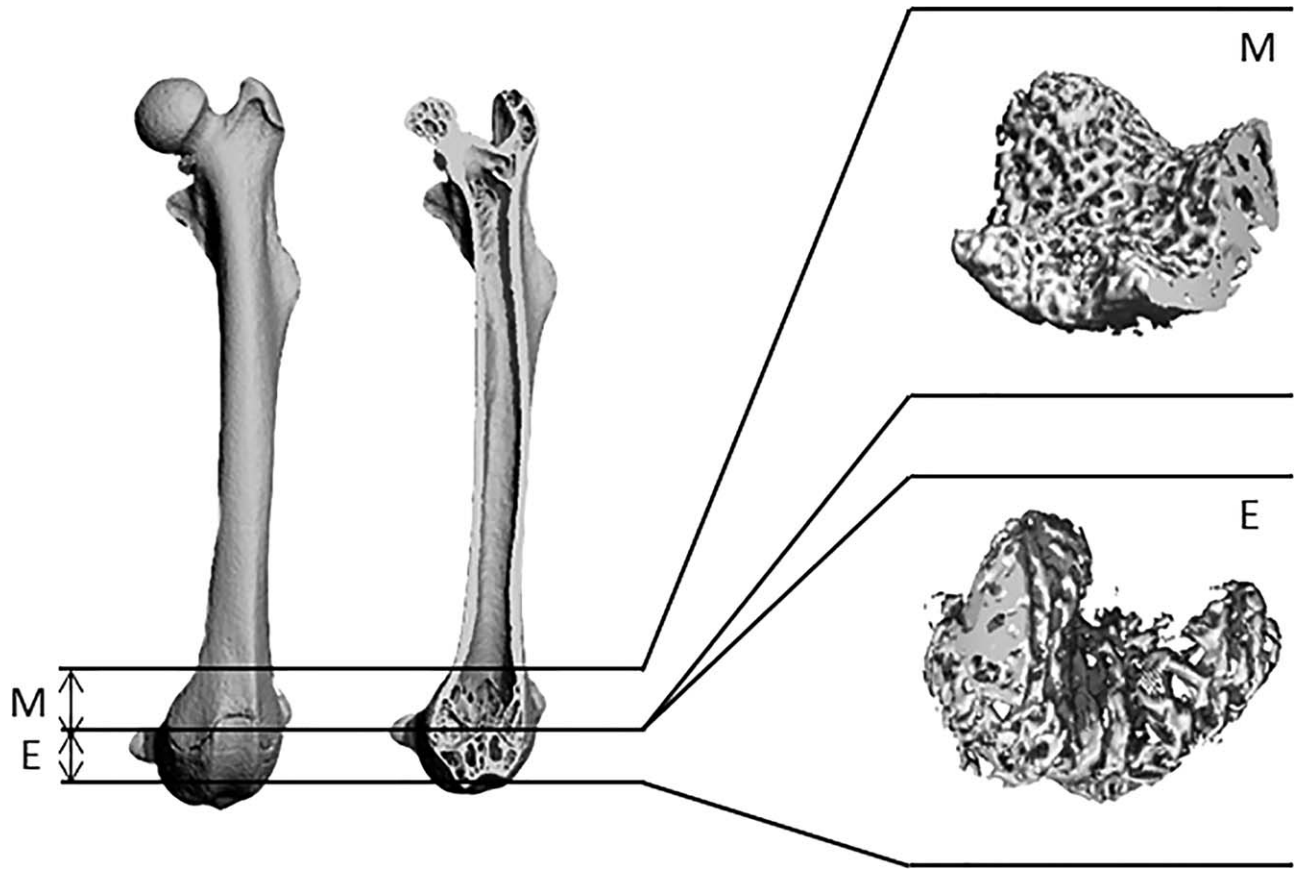

Fig. 2. Micro-computed tomography scans of a pocket mouse femur. Regions of interest include that metaphysis (M) and epiphysis (E).

failure. Strain was determined in equation (3) where d represents the measured displacement. Strain values were used to generate the stress–strain curve to determine elastic modulus (E) which is the slope of the linear region of the stress–strain curve.

$$\varepsilon = \frac{12 * c * d}{L^2} \tag{3}$$

Osteocyte Lacunar Properties

Osteocyte lacunar properties were quantified in femoral cross-sections ground to $\leq$90 μm. Entire cross-sections were imaged at 400× magnification with a Nikon Eclipse 80i microscope and Olympus DP71 camera. Lacunar measurements were made using the image analysis software Bioquant Osteo II (Nashville, TN). All osteocyte lacunae in focus in the field of view for each image were measured as previously described (Qing et al., 2012b; Wojda et al., 2016). Osteocyte lacunar area (Lc.Ar), lacunar density (Lc.Dn), and lacunar porosity (Lc.Por) were quantified. Osteocyte lacunar density was calculated as the total number of lacunae divided by the bone area and lacunar porosity was calculated as the total lacunar area divided by the bone area (McGee-Lawrence et al., 2011).

Trabecular Bone Properties

Prior to mechanical testing the distal 3 mm of the left femur was scanned and trabecular bone properties were assessed via micro-computed tomography (μCT) (SCANCO μCT 80 Medical, Switzerland). The bones were scanned at 70 kVp, 114 μA, and 8 watts at high resolution (10 μm voxel size). Trabecular bone properties were analyzed at a threshold of 180–220 for all of the trabecular bone in the entire epiphysis and metaphysis of the distal femur (Fig. 2); the number of slices analyzed in the epiphyses ranged from 103 to 173 and the number of slices analyzed in the metaphyses ranged from 81 to 148. Trabecular bone regions of interest were drawn ~500 μm inside the cortical shell using the SCANCO software, and care was taken to ensure the growth plate was not included in the analysis of trabecular properties. Properties obtained include bone volume fraction (BV/TV), material mineral density (Mat.Mn.Dn), trabecular thickness (Tb.Th), trabecular spacing (Tb.Sp) and trabecular number (Tb.N). Bone volume fraction is calculated as the trabecular bone area divided by the trabecular tissue area (McGee-Lawrence et al., 2011). Material mineral density is reported in units of milligrams of hydroxyapatite per unit volume (cubic centimeters). Trabecular thickness and spacing are reported in units of millimeters. Trabecular number is measured in units of #/mm (Bouxsein et al., 2010).

TABLE 1. Results from a one-way ANOVA of geometrical properties at the femur midshaft across all months of sample collection ($n = 136$).

Property	Mean ± SD	Max, Min	P
Ct.Th (μm)	137.6 ± 16.2	174.6, 89.1	0.0006[a]
Ct.Ar (mm^2)	0.299 ± 0.055	0.5, 0.193	<0.0001
I_{max} (mm^4)	0.031 ± 0.024	0.3, 0.015	0.1766

[a]Holm–Sidak test did not detect significant differences between months ($P > 0.002$).

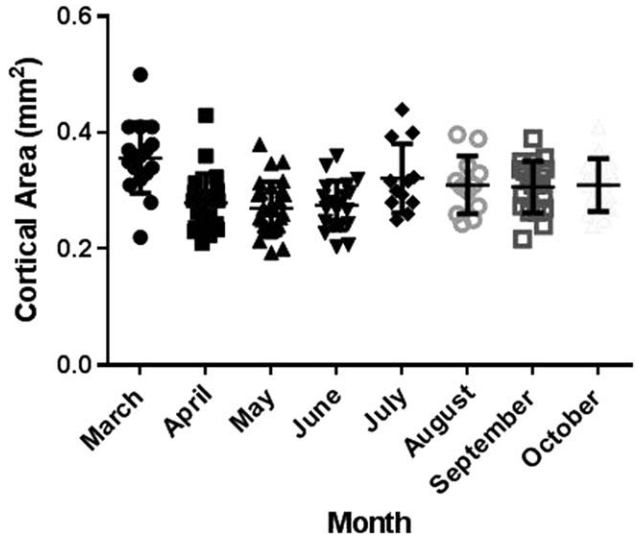

Fig. 3. Results of one-way ANOVA for cortical area ($n = 136$) showing significant differences across months ($P < 0.0001$). Post-hoc comparisons indicated cortical area was greater ($P < 0.001$) in March compared to April, May, and June.

Statistics

The objective of this study was to compare differences in bone properties of little pocket mice between the months they were active above ground (March to October). They did not enter the traps November to February, suggesting they spent that time period in hibernation. Not all analyses contained all 187 samples. For analysis of some properties (e.g., mechanical properties) a subset of the available samples were randomly selected. Some histological samples were lost during sample preparation and processing. A two-way ANOVA for each dependent variable was used to determine if month or sex significantly affected bone properties. Sex did not significantly ($P > 0.05$) affect any of the dependent bone variables except for trabecular thickness (Tb.Th) of the metaphysis. However, some months only had one female sample for metaphyseal Tb.Th. Thus, males and females were grouped together for the between month comparisons of the dependent bone variables using a one-way ANOVA. ANOVAs were considered statistically significant for $P \leq 0.05$. Because bone properties were compared from samples collected in 8 different months, significant ANOVAs were followed up with Holm-Sidak post-hoc multiple comparisons tests. A total of 28 comparisons were analyzed with significance for each comparison set to $P \leq 0.002$ according to the Sidak modification with $\alpha = 0.05$. SPSS Statistics software was used for statistical analyses.

TABLE 2. Results from a one-way ANOVA of mechanical properties from three-point bending tests across all months of sample collection ($n = 36$).

Property	Mean ± SD	Max, Min	P
σ_{ult} (MPa)	162 ± 55	270, 15	0.3868
u (mJ mm^{-3})	3.79 ± 1.58	6.76, 0.78	0.1975
E (MPa)	4.038 ± 1.210	7,003, 873	0.1593
U_f (J)	0.873 ± 0.296	1.39, 0.14	0.3357

TABLE 3. Results from a one-way ANOVA of osteocyte lacunar properties at the femur mid-diaphysis across all months of sample collection ($n = 108$)

Property	Mean ± SD	Max, Min	P
Lc.Ar (μm^2)	17.6 ± 3.1	34.9, 11.8	0.1878
Lc.Dn (#/mm^2)	303 ± 61	464, 179	0.1217
Lc.Por (%)	0.6 ± 0.9	8.5, 0.001	0.7411

RESULTS

The body mass of all 187 of the little pocket mice ranged from 5.33 to 9.89 g with an average body mass of 7.68 ± 0.79 g. Femur length of 150 samples ranged from 8.74 to 11.5 mm with an average length of 10.77 ± 0.49 mm. There were no differences in body mass ($p = 0.73$) or femur length ($P = 0.435$) between male and female mice. Body mass varied significantly with month ($P < 0.0001$), while femur length did not ($P = 0.103$). Body mass was greater in April (8.24 g) than in October (7.23 g).

Femoral Geometrical Properties

The maximum cross-sectional moment of inertia (I_{max}) at the mid-diaphysis of the femur was not different ($P = 0.1766$) between months (Table 1). However, cortical area (Ct.Ar) was higher ($P < 0.0001$) in March than in April, May and June (Fig. 3). All other comparisons of cortical area were not significant ($P \geq 0.002$). Cortical thickness (Ct.Th) was not different between months ($P \geq 0.002$).

Femoral Mechanical Properties

Femoral mechanical properties were not significantly different ($P \geq 0.1593$) between months (Table 2).

Osteocyte Lacunar Properties

Osteocyte lacunar properties were not different ($P \geq 0.1217$) across all months of sample collection (Table 3).

Trabecular Bone Properties

Epiphyseal trabecular bone properties showed no differences ($P \geq 0.1521$) between months (Table 4). Trabecular thickness (Tb.Th) of the metaphysis approached significance ($P = 0.0683$). Bone volume fraction (BV/TV)

TABLE 4. Results from a one-way ANOVA of trabecular bone microstructural properties in the distal femur epiphysis and metaphysis, measured by μCT across all months of sample collection ($n = 39$)

Bone region	Property	Mean ± SD	Max, Min	P
Epiphysis	BV/TV (%)	15.9 ± 2.4	21.8, 11.9	0.3765
	Tb.Th (mm)	0.041 ± 0.003	0.047, 0.034	0.1521
	Tb.Sp (mm)	0.278 ± 0.038	0.352, 0.196	0.1829
	Mat.Mn.Dn (mgHA/cm^3)	896 ± 55	1004, 740	0.3087
	Tb.N (#/mm)	3.7 ± 0.5	5.1, 2.9	0.5810
Metaphysis	BV/TV (%)	16.5 ± 7.0	34.0, 6.5	**0.0442**[a]
	Tb.Th (mm)	0.037 ± 0.0051	0.051, 0.029	0.0683
	Tb.Sp (mm)	0.217 ± 0.09	0.411, 0.064	**0.0390**
	Mat.Mn.Dn (mgHA/cm^3)	840 ± 67	977, 597	0.1935
	Tb.N (#/mm)	5.6 ± 2.7	14.9, 2.5	**0.0064**

[a]Holm–Sidak test did not detect significant differences between months ($P > 0.002$).

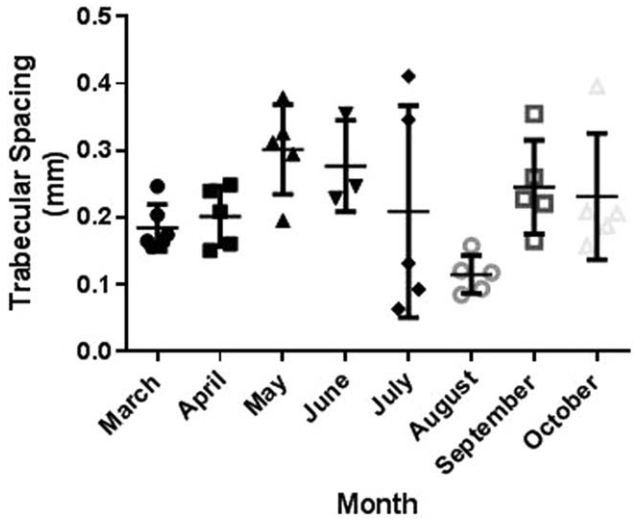

Fig. 4. Results of one-way ANOVA for trabecular spacing of the metaphysis (Tb.Sp) ($n = 39$) showing significant differences across months ($P = 0.0390$): Tb.Sp was greater ($P = 0.0009$) in May than August.

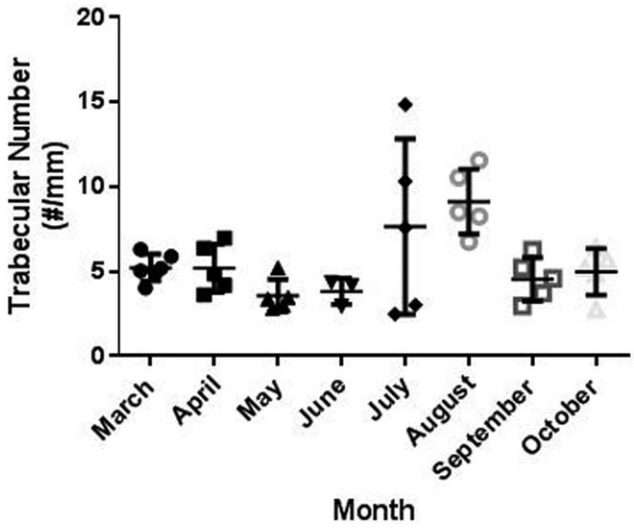

Fig. 5. Results of a one-way ANOVA for trabecular number of the metaphysis (Tb.N) ($n = 39$) showing significant changes ($P = 0.0064$): Tb.N was lower ($P = 0.0004$) in May than August.

of the metaphyseal region showed significance by ANOVA ($P = 0.0442$), but the post-hoc test comparisons found no differences ($P \geq 0.002$) between months. Metaphyseal trabecular spacing (Tb.Sp) showed differences ($P = 0.039$), but only between the months of May and August ($P = 0.0009$) (Fig. 4). Trabecular number (Tb.N) of the metaphysis also displayed differences ($P = 0.0004$) between May and August (Fig. 5). All other comparisons were not significant ($P \geq 0.002$).

DISCUSSION

Bone properties in hibernating mammals are commonly studied to understand how they are affected by periods of physical inactivity for comparison with other species (e.g., humans, mice, rats, and dogs) that experience bone loss during extended periods of disuse (Vico et al., 2000; Takata and Yasui, 2001). Both small and large hibernating mammals have demonstrated abilities to prevent disuse osteoporosis, but vary in how their properties are affected (Whalen et al., 1972; Steinberg et al., 1981; McGee-Lawrence et al., 2008; McGee et al., 2008; Utz et al., 2009; McGee-Lawrence et al., 2011; Doherty et al., 2012; Wojda et al., 2012; McGee-Lawrence et al., 2015; Doherty et al., 2016; Wojda et al., 2016). In addition to entering numerous cyclical torpor bouts interrupted by brief interbout arousals during the winter hibernation period, pocket mice are also able to enter torpor in the summer when challenged with extreme hot temperatures and food deficiency, even without cached food stores or excessive fat accumulation (Bartholomew and Cade, 1957; Bradley et al., 1975). This adaptation makes them appealing subjects to further investigate the impacts disuse has on skeletal properties in small hibernators. Understanding the impacts of these patterns on the skeletal system throughout the year is important for understanding how different hibernators have adapted to prevent disuse osteoporosis during prolonged periods of physical inactivity. Past studies have shown that large hibernators, such as bears preserve bone during hibernation through balanced bone resorption and formation (McGee et al., 2008; McGee-Lawrence et al., 2015). Some smaller species such as bats (Whalen et al., 1972) and golden hamsters (Steinberg et al., 1981) show bone loss during hibernation, but others such as squirrels (Utz et al., 2009; McGee-

Lawrence et al., 2011; Wojda et al., 2016) and marmots (Doherty et al., 2012; Wojda et al., 2012) preserve bone during hibernation. We did not find differences in bone properties between the pre-hibernation month of October and the post-hibernation month of March, suggesting bone properties are not affected by ~4 months of physical inactivity (Nov to Feb) during hibernation. We did find a few differences in bone properties between some of the months (March to October) in which little pocket mice are physically active above ground. This is possibly attributable to varying levels of physical activity (and therefore the mechanical stresses on bones) of little pocket mice throughout the year due to seasonal variations in temperature and food supply. However, because we were unable to determine the age of our samples, it is also possible that the differences in bone properties between some of the non-hibernating months were due to growth and development (McGee-Lawrence et al., 2011; Wojda et al., 2016).

It is known that little pocket mice spend most of their lives underground in burrow networks and undergo torpor during hibernation and possibly during summer estivation (Bartholomew and Cade, 1957; Kenagy, 1973). Because of this, it is unknown when the samples used in this study were collected relative to their most recent torpor bout. Because the animals were trapped in their natural habitat, the details on the number and length of torpor bouts and levels of physical activity are unknown. Little pocket mice did not enter our traps between November and February, suggesting they were inactive above ground and in a presumed state of hibernation since lab studies have shown that little pocket mice remain in their burrows during winter months, while undergoing short torpor bouts no longer than 96 hr (Kenagy, 1973). It is possible that the animals were physically active between torpor bouts and that food storage was adequate to prevent surfacing. However, hibernating rodents typically spend much of the interbout arousal period sleeping (Heller and Ruby, 2004). If the pocket mice were physically active between torpor bouts it is not clear how the brief period of physical activity would influence bone metabolism. It has been shown that rats lose significant bone mass after 4 weeks of hindlimb suspension and that the bone loss is not mitigated by daily exercise bouts on a treadmill (Shaw et al., 1987). Although the current study is lacking data for November–February, the study is valuable in understanding variation in bone properties in the months before and after winter hibernation and throughout the remainder of the year when the mice were physically active on the surface and entered traps. Samples were only collected over the course of 1 year and in one location, so it is possible that more variation in bone properties would be seen over multiple years due to various weather patterns. It is known that pocket mice live in burrow networks and are driven deeper underground due to changes in temperature (Kenagy, 1973), but temperature data was not available for this study. Thus, we cannot determine if monthly differences in bone properties are due to temperature and physical activity levels or growth and development.

The mechanical properties determined from three-point bending tests were maintained across the months of sample collection. These results are consistent with other hibernators that have been found to maintain mechanical strength of bones between pre- and post-hibernation periods (McGee-Lawrence et al., 2008, 2011; McGee et al., 2008; Utz et al., 2009; Doherty et al., 2012; Wojda et al., 2012). However, sample sizes were lower for mechanical properties than for geometrical properties and therefore comparisons for mechanical properties may have been underpowered. The maximum moment of inertia at the mid-shaft of the femur was maintained across all months, consistent with the preservation of bending stress. However, cross-sectional area did vary between months. Studies on lab mice have shown hind limb suspension decreases cortical bone area and suggest the change may be due to a reduction in growth (Simske et al., 1992). There was also a decrease in the cortical area of dog forelimbs, due to increases in bone remodeling, that were exposed to long term disuse by immobilization (Li et al., 2005). However, cortical bone changes were not found in other hibernation models (McGee et al., 2008; McGee-Lawrence et al., 2009, 2011; Doherty et al., 2012; Wojda et al., 2012; Wojda et al., 2016). Comparing prehibernation (October) to posthibernation (March) bone properties of little pocket mice in the present study, our findings were similar to those of other species that show no changes in macrostructural properties during winter hibernation (McGee et al., 2008; Utz et al., 2009; McGee-Lawrence et al., 2011, 2015; Doherty et al., 2012; Wojda et al., 2012Wojda et al., 2016). Little brown bats and golden hamsters showed possible loss in cortical thickness during hibernation (Whalen et al., 1972; Steinberg et al., 1981). Little pocket mice did not show changes in cortical thickness between pre- and post-hibernation months. However, it is possible that some changes in bone properties occurred between our collection time points and the times that animals entered or exited hibernation. Monthly differences in little pocket mouse cortical area could be related to the seasonal timing of reproductive activity. Similar to kangaroo rats, little pocket mice have been observed to be pregnant in mid- to late-winter with juveniles surfacing in spring (Kenagy, 1973). This reproductive pattern could account for smaller cortical area values in April, May and June than in March if mice are born in April, May and June.

Osteocyte lacunar properties in little pocket mouse cortical bone were not different between months. Previous studies conducted on osteocytic perilacunar remodeling in lab mice found that disuse did not affect lacunar area, but lactation caused an increase in osteocyte lacunar area that returned to normal 7 days post-lactation. Changes in lacunar areas are due to the ability of osteocytes to remove and replace their surrounding mineralized matrix during lactation, contributing to organismal calcium homeostasis (Qing et al., 2012). The lack of differences in lacunar properties between pre-hibernation and post-hibernation pocket mice suggest disuse due to physical inactivity did not affect lacunar properties, similar to findings in lab mice studies (Qing et al., 2012). It is unknown if any of the pocket mice collected for the present study were undergoing lactation, so we cannot comment on a possible role of osteocytic perilacunar remodeling in maintaining calcium homeostasis in lactating pocket mice. Because metabolism is suppressed during hibernation, there is likely low demand for ionized calcium and this may help explain the lack of changes that occurred in osteocytic lacunar properties

during hibernation. Pocket mice are too small to demonstrate intracortical bone remodeling, but hibernating bears have been shown to significantly suppress intracortical remodeling, probably due to the decrease in demand for ionized calcium and the need to conserve metabolic energy (McGee et al., 2008). Arctic and 13-lined ground squirrels also did not show changes in osteocytic lacunar area during hibernation, but they did have increased lacunar porosity during hibernation due to increased osteocyte lacunar density (McGee-Lawrence et al., 2011; Wojda et al., 2016). In those studies, the increase in lacunar density was associated with a decrease in osteoblast activity prompting terminal differentiation to osteocytes. However, in yellow-bellied marmots, a large hibernating rodent, osteocytic lacunar area, porosity, and density were lower post-hibernation compared to pre-hibernation. Clearly, more work is needed to elucidate the role that osteocytic perilacunar remodeling plays in maintaining calcium homeostasis in hibernators.

Trabecular bone architecture in the epiphysis appears to be preserved in pocket mice across the year. Trabecular bone properties of the epiphysis have also been shown to be maintained in hibernating 13-lined ground squirrels, marmots, and bears (McGee et al., 2008; McGee-Lawrence et al., 2011; Doherty et al., 2012; Wojda et al., 2012). However, some changes were observed in the metaphyseal region in trabecular number and spacing in little pocket mice. There were no differences between the pre- (October) and post-hibernation (March) months, suggesting winter hibernation did not affect trabecular bone properties of the metaphysis. However, there was a decrease in trabecular spacing between May and August with a corresponding increase in the number of trabeculae between the same months. These differences may also be attributed to growth and development of little pocket mice born in spring (April or May) and maturing by the late summer (August) (Kenagy, 1973). A future study on osteoblast and osteoclast activity in the metaphyseal region could help explain these observed changes in relation to seasonal temperature and growth cycles.

Overall, this study indicates that hibernation does not adversely affect the bone properties of little pocket mice. The few changes in bone properties detected between the months of March-October are possibly due to temperature, physical activity levels, and food collection, which would affect the amount of mechanical stresses put on the bones, but also may be due to growth and development. The seasonal changes observed in little pocket mouse cortical and trabecular bone properties are consistent with findings in arctic ground squirrels and the known periods of reproduction and growth of little pocket mice (Kenagy, 1973; Wojda et al., 2016). Past studies have shown that bone strength is preserved in several hibernating species, consistent with what was found in the present study. It appears that while larger hibernators, such as bears, can maintain bone microstructure, some smaller hibernators display some bone microstructure changes, likely due to differences in the physiological mechanisms between species that regulate bone and calcium metabolism. Future studies that analyze the effects of physical activity levels and seasonal temperature fluctuations of little pocket mice, of varying ages, on bone remodeling could be done to address the unknown causative factors for the monthly differences observed in bone properties.

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