

RESEARCH ARTICLE

Raccoon dog model shows preservation of bone during prolonged catabolism and reduced physical activity

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ABSTRACT

The raccoon dog (*Nyctereutes procyonoides*) is a promising animal model capable of preventing disuse-induced osteoporosis. Previous data suggest that this species resembles bears in the preservation of bone mass and biomechanical properties during prolonged passivity and catabolism. This longitudinal study examined the osteological properties of tibiae in farm-bred raccoon dogs that were either fed or fasted ($n=6$ per group) for a 10 week period. Peripheral quantitative computed tomography was utilized and plasma markers of bone turnover measured before fasting and at 9 weeks followed by mechanical testing (three-point bending), micro-computed tomography and Fourier transform infrared imaging at 10 weeks. Passive wintering with prolonged catabolism (body mass loss 32%) had no significant effects on bone mineralization, porosity or strength. The concentration of C-terminal telopeptide of type I collagen, indicative of bone resorption, increased in the plasma of the fasted raccoon dogs, while the bone formation markers were unchanged. The levels of 25-hydroxyvitamin D were reduced in the fasted animals. Based on these data, the preservation of bone in wintering raccoon dogs shares characteristics with that of bears with no apparent decrease in the formation of bone but increased resorption. To conclude, raccoon dogs were able to minimize bone loss during a 10 week period of catabolism and passivity.

KEY WORDS: Bone mineral density, Bone turnover markers, Catabolism, Fasting, Fourier transform infrared imaging, Hibernation, *Nyctereutes procyonoides*

INTRODUCTION

Osteopenia and osteoporosis are prevalent especially in postmenopausal women, and pose significant health and economic burdens (Siris et al., 2001). In addition to the decline in estrogen levels, disuse is one of the main inducers of bone loss,

eventually leading to osteoporosis (Lau and Guo, 2011). Reduced bone mass and deteriorated microarchitecture increase the risk of fractures, e.g. during long-term bed rest, paralysis or exposure to microgravity. This skeletal fragility results from unbalanced remodeling in which bone resorption typically increases while bone formation can decrease, leading to increased circulating calcium concentrations and excretion (Inoue et al., 2000; McGee-Lawrence et al., 2008). The loss is usually more pronounced in the trabecular bone than in the cortical bone, because of the larger surface area of the trabecular bone (McGee-Lawrence et al., 2008; Lau and Guo, 2011). With no effective treatment for disuse-induced osteoporosis, animal models that can unravel mechanisms to prevent this condition are urgently needed. Negative energy balance also reduces bone mineral density (BMD) and/or strength (Talbot et al., 2001; Sonne et al., 2009; Misra and Klibanski, 2011).

Previous studies suggest that small mammalian hibernators would not be able to prevent bone loss during overwintering although data remain inconclusive (McGee-Lawrence et al., 2008). In contrast to small hibernators, traditional laboratory rodents and humans, several bear (Ursidae) species that exhibit large-scale seasonal body mass (M_b) fluctuations and prolonged wintertime passivity, fasting and anuria have been shown to be highly resistant to bone mass loss even after several months of locomotory system disuse and constant catabolism. For instance, American black bears (*Ursus americanus*) preserve bone volume, mineral content, porosity and strength during their 5–7 month hibernation (Donahue et al., 2006b). In grizzly bears (*Ursus arctos horribilis*), porosity can be even lower in hibernating than in active individuals (McGee et al., 2008).

It has been demonstrated by using serum bone remodeling markers that bone resorption increases in hibernating bears while bone formation remains stable (McGee-Lawrence et al., 2008). Bone remodeling can decrease but bone loss could be prevented by balanced resorption and formation during inactivity. Bears may still lose some bone during hibernation but it could be rapidly recovered with remobilization after arousal (Donahue et al., 2003a,b, 2006b). Usually, a remobilization period 2–3 times longer than the duration of disuse is required for the full recovery of lost bone, e.g. in rats, but this does not apply to bears (McGee-Lawrence et al., 2008). Instead, they have been suggested to utilize various mechanisms to maintain the balance between bone formation and resorption during inactivity. One potential mechanism is calcium recycling via parathyroid hormone (Donahue et al., 2006a; McGee-Lawrence et al., 2009a,b). In addition, low-level mechanical stimulation (shivering, grooming, rolling over in the den) and limited (<20 min) daily physical activity instead of total immobility may contribute to bone mass preservation (Donahue et al., 2006b). While bears have provided valuable data for osteoporosis research, they are large animals that require special techniques to be examined safely. There also exist mouse models that are resistant to disuse-induced

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List of abbreviations

μ CT	micro-computed tomography
ALT	alanine aminotransferase
AP	alkaline phosphatase
AST	aspartate aminotransferase
ATR	attenuated total reflection
BMD	bone mineral density
FTIRI	Fourier transform infrared imaging
ICTP	C-terminal telopeptide of type I collagen
IP	inorganic phosphorus
M_b	body mass
PBS	phosphate-buffered saline
PINP	N-terminal propeptide of type I procollagen
pQCT	peripheral quantitative computed tomography
Vit D-25	25-hydroxyvitamin D

osteoporosis (Judex et al., 2004; Kondo et al., 2011). Mice are more easily reared and handled but sample volumes (e.g. blood) are necessarily smaller than in larger species.

A few years ago, we introduced the raccoon dog, *Nyctereutes procyonoides* (Gray 1834), model as a more practical species to study bone mass preservation during prolonged natural catabolism (Nieminen et al., 2011). Raccoon dogs are of moderate M_b (approximately 10 kg), easy to handle and reared commercially. Thus, experimental animals are readily available with established methods for anesthesia and blood sampling (Mustonen et al., 2004, 2007a,b, 2012). With similarities to bears, raccoon dogs display seasonal lethargy (hereafter, hibernation) with prolonged catabolism and reduced locomotion, moderately suppressed metabolic rate and slightly decreased body temperature (Mustonen et al., 2007a, 2012). Their M_b decreases by approximately 40% during hibernation, which can last up to 9 weeks in northern latitudes (Mustonen et al., 2007a). Their appetite also decreases over winter on farms (Korhonen, 1987), and fasted animals experience a reduction in their locomotor activity (Nieminen et al., 2002). We previously observed unchanged BMD and biomechanical properties during overwintering in wild specimens (Nieminen et al., 2011). However, the histological samples still revealed a decrease in the proportion of osteoid bone perimeter compared with mineralized bone perimeter, suggesting reduced bone formation during hibernation.

To assess the effects of prolonged fasting on osteological properties of raccoon dogs in a comprehensive manner, we performed a longitudinal study and followed changes in BMD, bone morphometric properties and bone metabolic markers from plasma in the same animals before and after prolonged catabolism. These data were complemented with detailed mechanical, microstructural and compositional analyses of bone at the end of fasting. We hypothesized that long-term food deprivation of a duration that occurs naturally (10 weeks) would not cause significant changes in osteological variables of raccoon dogs.

MATERIALS AND METHODS**Animals and fasting treatment**

The experimental protocol was approved by the National Animal Experiment Board (license no. ESLH-2009-08219/Ym-23) and the work was carried out in accordance with the European Communities Council Directive of 24 November 1986 (<http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:31986L0609>) and complied with the ARRIVE guidelines (Kilkenny et al., 2010). Twelve 7 month old farm-bred raccoon dogs (11 females and one male)

were randomly divided into two groups ($n=6$ per group). The sample size was determined with Mead's resource equation. The animals were housed individually in cages with nest boxes and straw bedding in roofed enclosures at natural ambient temperature and photoperiod at the Kannus Research Farm Luova Ltd, Finland (63.90833713N, 23.94056244E). The average M_b of all animals was 13.95 ± 0.41 kg on 7 December 2009, indicating that they had accumulated large fat reserves during autumn. The control animals were fed regularly with commercial fur animal diet while the other group was fasted for 10 weeks between 22 December 2009 and 1 March 2010. The fasted animals were housed in a separate shadow house to minimize stress during feeding time. All animals received water *ad libitum* and the control group was fasted overnight before blood sampling. The staff of the Research Farm monitored the health of the animals regularly.

Blood sampling and peripheral quantitative computed tomography (pQCT)

On 7 December 2009, all the raccoon dogs were transported to the Institute of Biomedicine, University of Oulu, Finland. They were anesthetized with intramuscular ketamine (5 mg kg^{-1}) and xylazine (2 mg kg^{-1}), weighed and blood samples were collected from a superficial vein of a hindleg into EDTA tubes and centrifuged at 1500 g for 15 min. The plasma samples were stored at -80°C . The raccoon dogs were placed on a custom-made bed for pQCT (XCT 960A with software v.5.2, Stratec Medizintechnik GmbH, Pforzheim, Germany) and the right hindlimb was extended backwards and fixed on the custom holder around the paw. A scout view was recorded based on which the reference line was set at the middle of the tibiotarsal joint. A tomographic slice with a voxel size of $590 \mu\text{m} \times 590 \mu\text{m} \times 1500 \mu\text{m}$ was measured 2.5 cm proximal to the joint. Trabecular bone was segmented first by detection of the outer contour using a threshold of 95 mg cm^{-3} , and pixels with BMD lower than 600 mg cm^{-3} in a 3×3 mask were considered as trabecular bone. The cortical bone was analyzed using a single threshold of 690 mg cm^{-3} to minimize the partial volume effect (Ward et al., 2005; Hangartner, 2007). Both periosteal and endosteal circumferences were defined by circular approximation. These procedures were conducted for the animals in a random order at 08:00 h–12:00 h by researchers unaware of the group assignment. The blood sampling and pQCT measurements were repeated using the same procedures at the University of Oulu after 9 weeks of food deprivation on 23 February 2010.

Tissue collection

At the end of the 10 week fast, the animals were killed with an electric shock according to the regulations of the Council of the European Union (<http://eur-lex.europa.eu/legal-content/EN/ALL/?uri=celex:31993L0119>). They were weighed, and the right hindlimb was removed as a whole and preserved within the surrounding tissues in diluted PBS buffer (Dulbecco's phosphate buffered saline $10\times$, Sigma-Aldrich, St Louis, MO, USA) at -80°C for further analysis.

Micro-computed tomography (μ CT)

The tibiae were wrapped in wet tissue and sealed with cling film and scanned with the SkyScan 1176 (Bruker microCT, Kontich, Belgium) with a $17.42 \mu\text{m}$ isotropic voxel size. The X-ray tube voltage was set to 80 kV, current to $300 \mu\text{A}$, and a copper–aluminium filter was used to reduce beam hardening. Projection images were collected every 0.3 deg over full rotation (360 deg) and frame averaging of 2 was used. The projections were reconstructed

with NRecon software (Bruker microCT) and standard morphometric parameters (Bouxsein et al., 2010) were calculated using CTAn software (Bruker microCT). Two volumes of interest were manually drawn into the distal trabecular bone and the mid-diaphyseal cortical bone. Both volumes of interest were 17 mm (1000 cross-sectional images) long and started either 35 mm (2000 images) or 1.7 mm (100 images) proximal to the distal tibial growth plate.

Biomechanical testing

After μ CT imaging, the tibiae were subjected to a three-point bending test by placing them on a metal support with a fixed span length of 85 mm and loading them until fracture at a single point mid-shaft with a constant loading speed of 0.155 mm s^{-1} using a universal testing machine (Instron 3366, Norwood, MA, USA; Nieminen et al., 2011). Well-established principles of bone biomechanical testing were applied (Jämsä et al., 1998, 1999). The acquired load–deformation curve was analyzed for force, deformation and energy at yield point and maximum load. Toughness was defined as the energy absorbed until the point where the bone broke in two separate pieces. In the linear part of the load–deformation curve, a first-degree polynomial line was fitted and stiffness was evaluated from the first term coefficient, i.e. the slope. Yield point was determined from the measured curve as the point where this fit was separated by 0.155 mm from the measured point. In addition, the whole loading test was recorded on video to monitor that the position of the bone remained stable during testing.

Fourier transform infrared imaging (FTIRI)

After biomechanical testing, one 3 mm-thick bone section was cut next to the fracture site with a diamond saw. The bone sample was polished using silicon carbide sandpaper with a decreasing grid size (800, 1200, 2400 and 4800). Polished surfaces were imaged with a Hyperion 3000 FTIRI microscope (Bruker Optics Inc., Billerica, MA, USA) using an attenuated total reflection (ATR) objective. The ATR crystal was compressed on the bone with a constant load, and spectral images were recorded with a focal plane array detector. Spatial and spectral resolutions were set to $20 \mu\text{m}$ and 8 cm^{-1} , respectively. Each spectrum between 800 and 3300 cm^{-1} was averaged 32 times and two spectral maps of 32×32 with a pixel size of $3.9 \mu\text{m}$ were collected from the anterior and posterior quarters of the bone section. Initial data analyses were conducted with CytoSpec software (Berlin, Germany) in the MATLAB environment (MathWorks Inc., Natick,

MA, USA). First, chemical maps for amide I, phosphate and carbonate concentrations were calculated by integrating areas under the curves between 1585 and 1720 cm^{-1} , 900 and 1200 cm^{-1} , and 850 and 890 cm^{-1} (Boskey and Pleshko Camacho, 2007). These maps were subsequently transferred to ImageJ software (National Institutes of Health, Bethesda, MD, USA), where they were thresholded in order to remove empty pixels (blood vessels). Finally, well-established parameters for bone composition (carbonate:amide I, mineral:matrix and carbonate:phosphate) were calculated from the thresholded spectral maps (Isaksson et al., 2010).

Biochemical analyses

The C-terminal telopeptide of type I collagen (ICTP), a bone resorption marker, and the intact N-terminal propeptide of type I procollagen (PINP), an indicator of bone formation (Kushida et al., 1995; Koivula et al., 2012), were determined from plasma with radioimmunoassay kits (Orion Diagnostica, Espoo, Finland). These analyses were validated for the raccoon dog with dilution series. The alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (AP) activities were analyzed by the International Federation of Clinical Chemistry method with the ADVIA 1800 Chemistry System (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA). The concentrations of inorganic phosphorus (IP) were measured in the form of phosphomolybdate by the spectrophotometric method (ADVIA 1800) and the 25-hydroxyvitamin D (Vit D-25) levels by the chemiluminescence immunometric method (Liaison[®], DiaSorin, Saluggia, Italy).

Statistical analyses

Comparisons between groups at different time points were performed with the Kruskal–Wallis analysis of variance (ANOVA; SPSS v.19 software package, IBM, Armonk, NY, USA). Regarding the variables that were analyzed only after euthanasia, the Mann–Whitney *U*-test was performed for the two study groups. Non-parametric tests were selected because of the relatively small sample size. $P < 0.05$ was considered statistically significant. The results are presented as means \pm s.e.

RESULTS

The initial pQCT measurements conducted before the fasting procedure did not show any differences in the measured variables between the study groups (Table 1). The fed animals lost $20.3 \pm 0.92\%$ of their M_b during the experiment (7 December – 1 March),

Table 1. Osteological peripheral quantitative computed tomography characteristics of the distal tibia of fed and fasted raccoon dogs before (December) and after (February) 9 week food deprivation of the fasted group

	December		February	
	Fed	Fasted	Fed	Fasted
Total mineral content (mg)	98 \pm 12	86 \pm 2	81 \pm 4	77 \pm 3
Total mineral density (mg cm^{-3})	624 \pm 24	633 \pm 34	596 \pm 21	585 \pm 32
Cortical mineral content (mg)	81 \pm 14	70 \pm 4	63 \pm 5	59 \pm 5
Cortical mineral density (mg cm^{-3})	749 \pm 12	772 \pm 15	756 \pm 19	781 \pm 16
Trabecular mineral content (mg)	28 \pm 4	26 \pm 3	30 \pm 2	29 \pm 2
Trabecular mineral density (mg cm^{-3})	440 \pm 18	428 \pm 20	421 \pm 16	404 \pm 21
Total area (mm^2)	156 \pm 16	137 \pm 7	136 \pm 7	133 \pm 5
Cortical area (mm^2)	109 \pm 19	91 \pm 5	83 \pm 8	75 \pm 5
Trabecular area (mm^2)	64 \pm 9	62 \pm 9	72 \pm 5	74 \pm 7
Cortical thickness (mm)	3.2 \pm 0.4	2.5 \pm 0.5	2.5 \pm 0.1	2.4 \pm 0.2
Periosteal circumference (mm)	42 \pm 2	41 \pm 2	41 \pm 1	40 \pm 1
Endosteal circumference (mm)	26 \pm 3	22 \pm 5	30 \pm 2	29 \pm 1
Polar moment of inertia (mm^4)	4326 \pm 863	3226 \pm 354	3219 \pm 354	3044 \pm 195
Moment of resistance (mm^3)	490 \pm 71	398 \pm 33	408 \pm 35	381 \pm 21

Data are means \pm s.e. There were no statistically significant differences between groups or time points.

while the decrease in the fasted animals was $31.9 \pm 0.77\%$ (Mann–Whitney U -test, $P=0.004$ between treatments). The pQCT measurements did not reveal any differences between the fed and fasted animals after 9 weeks of fasting (Table 1). Similarly, the three-point bending test conducted after 10 weeks did not reveal differences between the experimental groups (Table 2). In the μ CT analysis (Tables 3 and 4, Fig. 1), the specific bone surface area was greater in the fasted raccoon dogs in trabecular bone of the distal tibia after 10 weeks of fasting. The trabecular thickness and fractal dimension were higher in the fed group, while there were no differences in the other μ CT variables. Regarding FTIRI, there were no significant differences in the chemical composition of the tibiae (Table 5).

The fasted animals experienced a significant increase in ICTP values between December and February, with higher concentrations after 9 weeks of fasting compared with the fed controls, but the PINP concentrations were unresponsive (Table 6). As a result, the PINP:ICTP ratios decreased in the food-deprived group from December to February and were lower than in the fed group in February. The fasted raccoon dogs also experienced a reduction in Vit D-25 concentration from December to February. The ALT, AST and AP activities and IP concentration were unaffected by food deprivation. The seasonal trends in the plasma variables of the fed animals were non-significant.

DISCUSSION

The results of the present study indicate that bears are not unique in their ability to prevent bone loss during musculoskeletal disuse, as there was no clear deterioration in the osteological properties of the raccoon dogs in pQCT, three-point bending test, μ CT or FTIRI. Raccoon dogs are not classic deep hibernators, but their hibernation resembles that of bears, with moderate decreases in metabolic and heart rates and a small reduction in body temperature (Mustonen et al., 2007a, 2012; Kitao et al., 2009). It must be emphasized that the duration and continuity of hibernation are somewhat different between raccoon dogs and bears. Raccoon dogs are not completely immobile, fasting and anuric. Instead, they occasionally leave their dens during warmer periods to forage and to excrete waste. Their limb loading, however, clearly decreases from the snow-free season, with severely restricted movement and home range in midwinter. A fasting-induced reduction in physical activity can also be observed on farms (Nieminen et al., 2002). Excretion of calcium liberated from the bone as a consequence of reduced skeletal loading should lead to progressive bone loss in raccoon dogs, similar to that in small hibernators that arouse and excrete waste between their torpor bouts (McGee-Lawrence et al., 2008). Instead of excreting, bears can recycle calcium back into the skeleton by increased renal

Table 2. Variables related to bone strength of the distal tibia of raccoon dogs in March after 10 weeks of feeding or fasting

	Fed	Fasted
Stiffness (N mm^{-1})	317 \pm 17	303 \pm 18
D_{yield} (mm)	1.2 \pm 0.1	1.3 \pm 0.1
F_{yield} (N)	371 \pm 23	382 \pm 24
E_{yield} (mJ)	225 \pm 21	252 \pm 27
D_{max} (mm)	3.7 \pm 0.4	3.4 \pm 0.3
F_{max} (N)	653 \pm 31	638 \pm 33
E_{max} (J)	2.2 \pm 0.7	1.5 \pm 0.2
Toughness (J)	2.4 \pm 0.2	2.5 \pm 0.2

Data on stiffness, toughness, deformation (D), force (F) and energy (E) at yield point and maximum load are means \pm s.e. There were no statistically significant differences between groups.

Table 3. Results of 2D micro-computed tomography analysis of cortical bone of the mid-diaphyseal tibia of raccoon dogs in March after 10 weeks of feeding or fasting

	Fed	Fasted
Tissue mineral density (mg cm^{-3})	1308 \pm 6.3	1325 \pm 8.1
Cross-sectional bone area (mm^2)	44.2 \pm 1.81	43.5 \pm 1.87
Cross-sectional medullary area (mm^2)	17.8 \pm 1.20	17.6 \pm 1.09
Periosteal perimeter (mm)	31.1 \pm 0.77	31.4 \pm 0.49
Endosteal perimeter (mm)	18.0 \pm 0.84	18.8 \pm 1.20
Polar moment of inertia (mm^4)	583 \pm 47.7	566 \pm 35.9
Eccentricity	0.47 \pm 0.048	0.52 \pm 0.014
Cortical thickness (mm)	1.80 \pm 0.042	1.74 \pm 0.082

Data are means \pm s.e. There were no statistically significant differences between groups.

reabsorption via parathyroid hormone (Donahue et al., 2006a), but it is not yet known whether raccoon dogs can employ a similar strategy.

Disuse is characterized by an imbalance between bone resorption and bone formation (Lau and Guo, 2011). It seems that the bear models for bone preservation also experience increased resorption versus formation (Donahue et al., 2003a,b, 2006b). This should eventually lead to net bone loss; however, this is not usually observed in bears. In our study, there were no significant changes in the longitudinal structural and densitometric bone parameters. Moreover, the biomechanical properties of bone were similar between the fed and fasted groups at the end of the study. However, high-resolution μ CT, which could only be conducted at the later time point, indicated decreased trabecular thickness in the fasted group, while the net trabecular bone volume fraction was not significantly different. The plasma markers of bone remodeling indicated increased resorption (ICTP) but stable bone formation (PINP, AP) in the fasted raccoon dogs. Previous histological data point to a reduction of bone formation in wintering raccoon dogs (Nieminen et al., 2011). The present results suggest a slight imbalance in bone remodeling with net bone loss, but it is not yet known whether raccoon dogs only minimize bone loss during passivity or whether they would also be able to recover their bone properties after remobilization (see Stevenson et al., 2009, for *Myodes rutilus* voles). The paradox of the bone resorption and formation imbalance while preserving bone remains unresolved in both bears and raccoon dogs.

Table 4. Results of 3D micro-computed tomography analysis of trabecular bone of the distal tibia of raccoon dogs in March after 10 weeks of feeding or fasting

	Fed	Fasted
Bone volume (mm^3)	426 \pm 43.3	370 \pm 17.8
Trabecular bone fraction (%)	26.4 \pm 1.56	22.9 \pm 1.52
Bone surface (mm^2)	8488 \pm 762.0	8328 \pm 488.1
Specific bone surface (mm^{-1})	20.1 \pm 0.31	22.5 \pm 0.48*
Bone surface density (mm^{-1})	5.3 \pm 0.27	5.2 \pm 0.36
Trabecular thickness (mm)	0.17 \pm 0.002	0.15 \pm 0.002*
Trabecular separation (mm)	0.55 \pm 0.037	0.64 \pm 0.138
Trabecular number (mm^{-1})	1.59 \pm 0.087	1.51 \pm 0.107
Trabecular pattern factor (mm^{-1})	-0.13 \pm 0.794	0.78 \pm 0.474
Structure model index (a.u.)	0.61 \pm 0.141	0.80 \pm 0.092
Degree of anisotropy (a.u.)	2.09 \pm 0.083	2.12 \pm 0.035
Fractal dimension (a.u.)	2.52 \pm 0.016	2.47 \pm 0.010*
Euler number (a.u.)	-18,716 \pm 2710.2	-19,453 \pm 2949.6
Connectivity (a.u.)	28,353 \pm 3446.9	30,166 \pm 2664.8
Connectivity density (mm^{-3})	17.5 \pm 1.45	18.7 \pm 1.79

Data are means \pm s.e. a.u., arbitrary units. *Statistically significant difference between experimental groups (Mann–Whitney U -test, $P<0.05$).

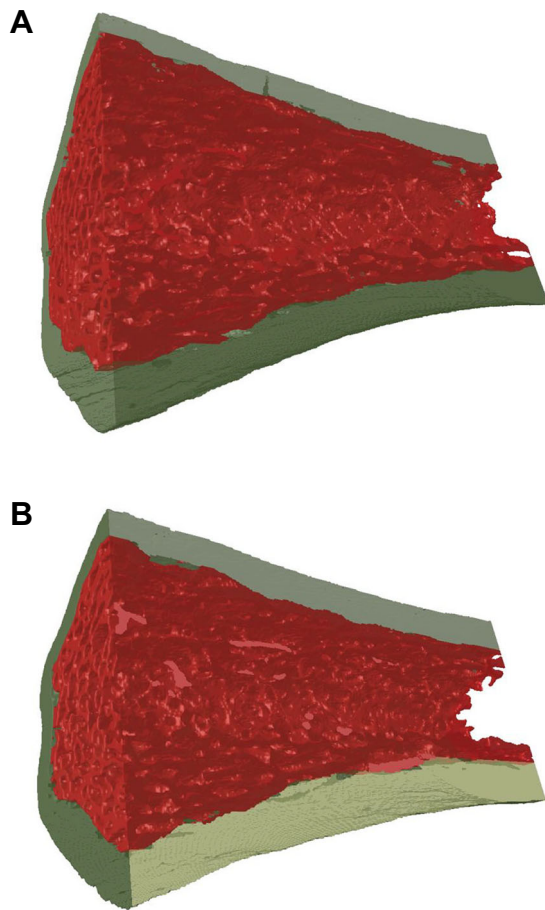


Fig. 1. 3D visualization of cortical (gray) and trabecular (red) bone in the distal tibia. The structure is similar for a fed (A) and a fasted (B) raccoon dog in March after 10 weeks of feeding or fasting.

Several hormones participate in bone remodeling and they could possibly play roles in the preservation of bone mass in raccoon dogs. High concentrations of glucocorticoids and thyroid hormones have deleterious effects on bone by inducing resorption and bone loss (Manelli and Giustina, 2000; Gorke et al., 2013). However, the levels of cortisol and tri/tetraiodothyronine decrease in raccoon dogs during hibernation (Asikainen et al., 2004; Mustonen et al., 2004), and these changes may help prevent disuse-induced osteoporosis. Growth hormone, in contrast, remains high in wintering raccoon dogs (Nieminen et al., 2002), with potential anabolic effects on bone (Ohlsson et al., 1998). The increasing sex steroid levels towards the mating season (Asikainen et al., 2003) could also promote the maintenance of bone mass (Horstman et al., 2012). Wintertime fasting does not affect the plasma leptin concentrations of raccoon

Table 5. Fourier transform infrared imaging results of the distal tibia of raccoon dogs in March after 10 weeks of feeding or fasting

	Anterior quarter		Posterior quarter	
	Fed	Fasted	Fed	Fasted
Carbonate:amide I	0.17±0.008	0.17±0.008	0.17±0.008	0.16±0.008
Mineral:matrix	10.0±0.32	9.8±0.16	9.8±0.29	9.3±0.23
Carbonate:phosphate	0.017±0.001	0.017±0.001	0.018±0.001	0.018±0.001

Data are means±s.e. There were no statistically significant differences between groups.

Table 6. Plasma variables of fed and fasted raccoon dogs before (December) and after (February) 9 week food deprivation of the fasted group

	December		February	
	Fed	Fasted	Fed	Fasted
PINP ($\mu\text{g l}^{-1}$)	2.6±0.2	2.4±0.3	2.1±0.4	2.2±0.4
ICTP ($\mu\text{g l}^{-1}$)	13.9±1.3	13.9±1.6	8.8±0.8	22.0±1.9* [‡]
PINP:ICTP	0.19±0.014	0.19±0.023	0.23±0.044	0.10±0.017* [‡]
AP (U l^{-1})	2.9±0.8	2.8±0.6	2.3±0.4	4.2±1.2
ALT (U l^{-1})	62±8	61±9	60±6	40±8
AST (U l^{-1})	50±4	50±6	64±10	48±5
Vit D-25 (nmol l^{-1})	188±12	199±16	148±9	105±8 [‡]
IP (mmol l^{-1})	1.37±0.05	1.42±0.07	1.27±0.06	1.34±0.03

PINP, intact N-terminal propeptide of type I procollagen; ICTP, C-terminal telopeptide of type I collagen; AP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Vit D-25, 25-hydroxyvitamin D; IP, inorganic phosphorus.

Data are means±s.e. *Significant difference from the fed group at the same time point (Kruskal–Wallis ANOVA, $P<0.05$). [‡]Significant difference from the previous values of the same experimental group (Kruskal–Wallis ANOVA, $P<0.05$).

dogs (Nieminen et al., 2002; Kinnunen et al., 2015), whereas in denning bears the leptin levels increase or remain stable (Donahue et al., 2006a; Seger et al., 2011). It has been suggested that the anti-osteogenic effects of leptin are mediated by noradrenaline that is released through the activation of the sympathetic nervous system and acts on the β_2 -adrenergic receptors on osteoblasts (Eleftheriou et al., 2004; He et al., 2013). It is unclear whether the persisting leptin concentrations would have an inhibitory effect on bone formation in wintering raccoon dogs. The combined actions of different endocrine signals are presumably complex. For instance, denning bears experience increased serum cortisol levels (Donahue et al., 2003a, b), which could as a single finding indicate bone loss. Interestingly, the levels of sclerostin, which decreases bone formation, can be elevated in hibernating bears (Seger et al., 2011). Sclerostin antibody treatment is being investigated for its potential to prevent unloading-induced osteoporosis (Qin et al., 2015).

Although non-significant, the ICTP concentrations of the fed raccoon dogs showed lower values in February than in December, while the levels clearly increased in the fasted group. Raccoon dogs decrease their food intake voluntarily in winter despite food being offered regularly (Korhonen, 1987). Their physical activity also decreases seasonally (Korhonen, 1988), although fed animals could be physically more active than those subjected to food deprivation (Nieminen et al., 2002). The combination of these issues should be taken into account when interpreting the results. In the fed group, there was also a decreasing trend in other markers of bone turnover, PINP and AP, indicating reduced bone formation. While all these markers of bone turnover tended to reduce in the fed animals, the balance (PINP:ICTP) was relatively stable, with slightly higher values in February. We suggest that with a seasonal decrease in food intake and activity, the fed animals still maintained balanced resorption and formation. This has some similarities to human observations, with formation and resorption markers increasing with physical activity (Thorsen et al., 1997). In contrast, the fasted animals were in the process of shifting the bone turnover balance towards resorption.

The Vit D-25 concentrations generally reflect the vitamin D status of the individual and can be used as an indicator of bone health (Holick, 2007). In denning bears, the Vit D-25 levels increased (Seger et al., 2011) and they could have participated in the prevention of bone loss. This is clearly different from the situation in

the raccoon dogs. We found decreased Vit D-25 concentrations during wintering in both study groups but a more pronounced reduction in the fasted animals. The nearly 50% lower values were, however, not associated with osteopenia but could probably be related to reduced vitamin D intake (Holick, 2007).

Previous studies have shown that raccoon dogs are very resistant to starvation and can fast for 8 weeks without the need for stimulated proteolysis as an energy source (Mustonen et al., 2004). No deleterious health effects have been documented during prolonged fasting regarding several organ systems (Mustonen et al., 2007b). In the present study, we verified this by monitoring plasma transaminase activities, increased levels of which would indicate liver cell damage (Rouvinen-Watt et al., 2010). The activities were not elevated in the fasted raccoon dogs, and the steady rate of M_b loss confirmed that the animals remained in phase II of fasting with fat mobilization (Mustonen et al., 2004). It can be summarized that, in addition to skeletal muscle function (Kinnunen et al., 2015), raccoon dogs can effectively preserve bone mass during wintertime food deprivation and immobility.

Based on our current results using pQCT, three-point bending test, μ CT, FTIRI and plasma biochemistry, the preservation of bone in wintering raccoon dogs shares similarities with that of bears. The present data provide further support that raccoon dogs are able to prevent bone loss during long-term catabolism and passivity, even though the marker of bone resorption increased and the markers related to bone formation remained stable. This imbalance would eventually lead to bone loss if the catabolic state were to continue longer. Immobilization studies are required in the future to verify the prevention of bone loss in raccoon dogs. Better understanding of the mechanisms for the preservation of bone in carnivore models can potentially lead to improved treatment of age- and disuse-induced osteoporosis in humans.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.-M.J.M., M.A.J.F., T.J.J., J.K.T., T.P.N.; Methodology: M.A.J.F., K.S.P., T.J.J., S.S., J.K.T.; Validation: A.-M.J.M., M.A.J.F., K.S.P., S.S.; Formal analysis: M.A.J.F., S.S., T.P.N.; Investigation: A.-M.J.M., M.A.J.F., S.S., T.P.N.; Resources: M.A.J.F., K.S.P., T.J.J., S.S., J.K.T., T.P.N.; Writing - original draft: A.-M.J.M.; Writing - review & editing: A.-M.J.M., M.A.J.F., K.S.P., T.J.J., S.S., J.K.T., T.P.N.; Visualization: M.A.J.F., T.P.N.; Supervision: A.-M.J.M., T.J.J., J.K.T., T.P.N.; Project administration: T.P.N.; Funding acquisition: M.A.J.F.

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References

- Asikainen, J., Mustonen, A.-M., Hyvärinen, H. and Nieminen, P. (2003). Seasonal reproductive endocrine profile of the raccoon dog (*Nyctereutes procyonoides*)—effects of melatonin and food deprivation. *J. Exp. Zool.* **299A**, 180-187.
- Asikainen, J., Mustonen, A.-M., Hyvärinen, H. and Nieminen, P. (2004). Seasonal physiology of the wild raccoon dog (*Nyctereutes procyonoides*). *Zool. Sci.* **21**, 385-391.
- Boskey, A. and Pleshko Camacho, N. (2007). FT-IR imaging of native and tissue-engineered bone and cartilage. *Biomaterials* **28**, 2465-2478.
- Bouxsein, M. L., Boyd, S. K., Christiansen, B. A., Goldberg, R. E., Jepsen, K. J. and Müller, R. (2010). Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. *J. Bone Miner. Res.* **25**, 1468-1486.
- Donahue, S. W., Vaughan, M. R., Demers, L. M. and Donahue, H. J. (2003a). Bone formation is not impaired by hibernation (disuse) in black bears *Ursus americanus*. *J. Exp. Biol.* **206**, 4233-4239.
- Donahue, S. W., Vaughan, M. R., Demers, L. M. and Donahue, H. J. (2003b). Serum markers of bone metabolism show bone loss in hibernating bears. *Clin. Orthop. Relat. Res.* **408**, 295-301.
- Donahue, S. W., Galley, S. A., Vaughan, M. R., Patterson-Buckendahl, P., Demers, L. M., Vance, J. L. and McGee, M. E. (2006a). Parathyroid hormone may maintain bone formation in hibernating black bears (*Ursus americanus*) to prevent disuse osteoporosis. *J. Exp. Biol.* **209**, 1630-1638.
- Donahue, S. W., McGee, M. E., Harvey, K. B., Vaughan, M. R. and Robbins, C. T. (2006b). Hibernating bears as a model for preventing disuse osteoporosis. *J. Biomech.* **39**, 1480-1488.
- Eleftheriou, F., Takeda, S., Ebihara, K., Magre, J., Patano, N., Kim, C. A., Ogawa, Y., Liu, X., Ware, S. M., Craigen, W. J. et al. (2004). Serum leptin level is a regulator of bone mass. *Proc. Natl. Acad. Sci. USA* **101**, 3258-3263.
- Gorka, J., Taylor-Gjevve, R. M. and Arnason, T. (2013). Metabolic and clinical consequences of hyperthyroidism on bone density. *Int. J. Endocrinol.* **2013**, 638727.
- Hangartner, T. N. (2007). Thresholding technique for accurate analysis of density and geometry in QCT, pQCT and μ CT images. *J. Musculoskelet. Neuronal Interact.* **7**, 9-16.
- He, J.-Y., Zheng, X.-F. and Jiang, L.-S. (2013). Autonomic control of bone formation: its clinical relevance. *Handb. Clin. Neurol.* **117**, 161-171.
- Holick, M. F. (2007). Vitamin D deficiency. *N. Engl. J. Med.* **357**, 266-281.
- Horstman, A. M., Dillon, E. L., Urban, R. J. and Sheffield-Moore, M. (2012). The role of androgens and estrogens on healthy aging and longevity. *J. Gerontol. A Biol. Sci. Med. Sci.* **67**, 1140-1152.
- Inoue, M., Tanaka, H., Moriwake, T., Oka, M., Sekiguchi, C. and Seino, Y. (2000). Altered biochemical markers of bone turnover in humans during 120 days of bed rest. *Bone* **26**, 281-286.
- Isaksson, H., Turunen, M. J., Rieppo, L., Saarakkala, S., Tamminen, I. S., Rieppo, J., Kröger, H. and Jurvelin, J. S. (2010). Infrared spectroscopy indicates altered bone turnover and remodeling activity in renal osteodystrophy. *J. Bone Miner. Res.* **25**, 1360-1366.
- Jämsä, T., Tuukkanen, J. and Jalovaara, P. (1998). Femoral neck strength of mouse in two loading configurations: method evaluation and fracture characteristics. *J. Biomech.* **31**, 723-729.
- Jämsä, T., Koivukangas, A., Ryhänen, J., Jalovaara, P. and Tuukkanen, J. (1999). Femoral neck is a sensitive indicator of bone loss in immobilized hind limb of mouse. *J. Bone Miner. Res.* **14**, 1708-1713.
- Judex, S., Garman, R., Squire, M., Busa, B., Donahue, L.-R. and Rubin, C. (2004). Genetically linked site-specificity of disuse osteoporosis. *J. Bone Miner. Res.* **19**, 607-613.
- Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M. and Altman, D. G. (2010). Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol.* **8**, e1000412.
- Kinnunen, S., Mänttari, S., Herzog, K.-H., Nieminen, P., Mustonen, A.-M. and Saarela, S. (2015). Maintenance of skeletal muscle energy homeostasis during prolonged wintertime fasting in the raccoon dog (*Nyctereutes procyonoides*). *J. Comp. Physiol. B* **185**, 435-445.
- Kitao, N., Fukui, D., Hashimoto, M. and Osborne, P. G. (2009). Overwintering strategy of wild free-ranging and enclosure-housed Japanese raccoon dogs (*Nyctereutes procyonoides albus*). *Int. J. Biometeorol.* **53**, 159-165.
- Koivula, M.-K., Risteli, L. and Risteli, J. (2012). Measurement of aminoterminal propeptide of type I procollagen (PINP) in serum. *Clin. Biochem.* **45**, 920-927.
- Kondo, H., Ezura, Y., Nakamoto, T., Hayata, T., Notomi, T., Sorimachi, H., Takeda, S. and Noda, M. (2011). MURF1 deficiency suppresses unloading-induced effects on osteoblasts and osteoclasts to lead to bone loss. *J. Cell. Biochem.* **112**, 3525-3530.
- Korhonen, H. (1987). Energy metabolism of raccoon dog (*Nyctereutes procyonoides*, Gray 1834): applied perspective to common farming practices. *PhD thesis*, University of Kuopio, Kuopio, Finland.
- Korhonen, H. (1988). Regulation of energy economy in raccoon dogs and blue foxes: a process of dynamic interactions. *Comp. Biochem. Physiol. A Comp. Physiol.* **91A**, 263-268.
- Kushida, K., Takahashi, M., Kawana, K. and Inoue, T. (1995). Comparison of markers for bone formation and resorption in premenopausal and postmenopausal subjects, and osteoporosis patients. *J. Clin. Endocrinol. Metab.* **80**, 2447-2450.
- Lau, R. Y.-c. and Guo, X. (2011). A review on current osteoporosis research: with special focus on disuse bone loss. *J. Osteoporos.* **2011**, 293808.
- Manelli, F. and Giustina, A. (2000). Glucocorticoid-induced osteoporosis. *Trends Endocrinol. Metab.* **11**, 79-85.
- McGee, M. E., Maki, A. J., Johnson, S. E., Nelson, O. L., Robbins, C. T. and Donahue, S. W. (2008). Decreased bone turnover with balanced resorption and formation prevent cortical bone loss during disuse (hibernation) in grizzly bears (*Ursus arctos horribilis*). *Bone* **42**, 396-404.
- McGee-Lawrence, M. E., Carey, H. V. and Donahue, S. W. (2008). Mammalian hibernation as a model of disuse osteoporosis: the effects of physical inactivity on bone metabolism, structure, and strength. *Am. J. Physiol.* **295**, R1999-R2014.
- McGee-Lawrence, M. E., Wojda, S. J., Barlow, L. N., Drummer, T. D., Bunnell, K., Auger, J., Black, H. L. and Donahue, S. W. (2009a). 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. *J. Biomech.* **42**, 1378-1383.

- McGee-Lawrence, M. E., Wojda, S. J., Barlow, L. N., Drummer, T. D., Castillo, A. B., Kennedy, O., Condon, K. W., Auger, J., Black, H. L., Nelson, O. L. et al. (2009b). Grizzly bears (*Ursus arctos horribilis*) and black bears (*Ursus americanus*) prevent trabecular bone loss during disuse (hibernation). *Bone* **45**, 1186-1191.
- Misra, M. and Klibanski, A. (2011). Bone health in anorexia nervosa. *Curr. Opin. Endocrinol. Diabetes Obes.* **18**, 376-382.
- Mustonen, A.-M., Nieminen, P., Puukka, M., Asikainen, J., Saarela, S., Karonen, S.-L., Kukkonen, J. V. K. and Hyvärinen, H. (2004). Physiological adaptations of the raccoon dog (*Nyctereutes procyonoides*) to seasonal fasting-fat and nitrogen metabolism and influence of continuous melatonin treatment. *J. Comp. Physiol. B* **174**, 1-12.
- Mustonen, A.-M., Asikainen, J., Kauhala, K., Paakkonen, T. and Nieminen, P. (2007a). Seasonal rhythms of body temperature in the free-ranging raccoon dog (*Nyctereutes procyonoides*) with special emphasis on winter sleep. *Chronobiol. Int.* **24**, 1095-1107.
- Mustonen, A.-M., Käkälä, R., Käkälä, A., Pyykönen, T., Aho, J. and Nieminen, P. (2007b). Lipid metabolism in the adipose tissues of a carnivore, the raccoon dog, during prolonged fasting. *Exp. Biol. Med.* **232**, 58-69.
- Mustonen, A.-M., Lempiäinen, T., Aspelund, M., Hellstedt, P., Ikonen, K., Itämies, J., Vähä, V., Erkinaro, J., Asikainen, J., Kunnasranta, M. et al. (2012). Application of change-point analysis to determine winter sleep patterns of the raccoon dog (*Nyctereutes procyonoides*) from body temperature recordings and a multi-faceted dietary and behavioral study of wintering. *BMC Ecol.* **12**, 27.
- Nieminen, P., Mustonen, A.-M., Asikainen, J. and Hyvärinen, H. (2002). Seasonal weight regulation of the raccoon dog (*Nyctereutes procyonoides*): interactions between melatonin, leptin, ghrelin, and growth hormone. *J. Biol. Rhythms* **17**, 155-163.
- Nieminen, P., Finnilä, M. A. J., Tuukkanen, J., Jämsä, T. and Mustonen, A.-M. (2011). Preservation of bone mass and biomechanical properties during winter sleep—the raccoon dog (*Nyctereutes procyonoides*) as a novel model species. *Bone* **48**, 878-884.
- Ohlsson, C., Bengtsson, B.-Å., Isaksson, O. G. P., Andreassen, T. T. and Słotweg, M. C. (1998). Growth hormone and bone. *Endocr. Rev.* **19**, 55-79.
- Qin, W., Li, X., Peng, Y., Harlow, L. M., Ren, Y., Wu, Y., Li, J., Qin, Y., Sun, J., Zheng, S. et al. (2015). Sclerostin antibody preserves the morphology and structure of osteocytes and blocks the severe skeletal deterioration after motor-complete spinal cord injury in rats. *J. Bone Miner. Res.* **30**, 1994-2004.
- Rouvinen-Watt, K., Mustonen, A.-M., Conway, R., Pal, C., Harris, L., Saarela, S., Strandberg, U. and Nieminen, P. (2010). Rapid development of fasting-induced hepatic lipidosis in the American mink (*Neovison vison*): effects of food deprivation and re-alimentation on body fat depots, tissue fatty acid profiles, hematology and endocrinology. *Lipids* **45**, 111-128.
- Seeger, R. L., Cross, R. A., Rosen, C. J., Causey, R. C., Gundberg, C. M., Carpenter, T. O., Chen, T. C., Halteman, W. A., Holick, M. F., Jakubas, W. J. et al. (2011). Investigating the mechanism for maintaining eucalcemia despite immobility and anuria in the hibernating American black bear (*Ursus americanus*). *Bone* **49**, 1205-1212.
- Siris, E. S., Miller, P. D., Barrett-Connor, E., Faulkner, K. G., Wehren, L. E., Abbott, T. A., Berger, M. L., Santora, A. C. and Sherwood, L. M. (2001). Identification and fracture outcomes of undiagnosed low bone mineral density in postmenopausal women: results from the National Osteoporosis Risk Assessment. *JAMA* **286**, 2815-2822.
- Sonne, C., Wolkers, H., Rigét, F. F., Jensen, J.-E. B., Teilmann, J., Jenssen, B. M., Fuglei, E., Ahlstrøm, Ø., Dietz, R., Muir, D. C. G. et al. (2009). Mineral density and biomechanical properties of bone tissue from male Arctic foxes (*Vulpes lagopus*) exposed to organochlorine contaminants and emaciation. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **149C**, 97-103.
- Stevenson, K. T., van Tets, I. G. and Chon, D. Y. (2009). Making no bones about it: bone mineral density changes seasonally in a nonhibernating Alaskan rodent. *J. Mammal.* **90**, 25-31.
- Talbott, S. M., Cifuentes, M., Dunn, M. G. and Shapses, S. A. (2001). Energy restriction reduces bone density and biomechanical properties in aged female rats. *J. Nutr.* **131**, 2382-2387.
- Thorsen, K., Kristoffersson, A., Hultdin, J. and Lorentzon, R. (1997). Effects of moderate endurance exercise on calcium, parathyroid hormone, and markers of bone metabolism in young women. *Calcif. Tissue Int.* **60**, 16-20.
- Ward, K. A., Adams, J. E. and Hangartner, T. N. (2005). Recommendations for thresholds for cortical bone geometry and density measurement by peripheral quantitative computed tomography. *Calcif. Tissue Int.* **77**, 275-280.