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

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Summary statement

The raccoon dog is a promising model of osteoporosis prevention similar to bears. We observed that despite profound catabolism and relative immobility it does not lose bone density or strength

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/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 reduced in the fasted animals. Based on these data, the preservation of bone in wintering raccoon dogs shares characteristics 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.

Keywords: bone mineral density, catabolism, fasting, Fourier transform infrared imaging, hibernation, markers of bone turnover, *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 reasons that induce 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). Compared to cortical bone, the loss is usually more pronounced in trabecular bone due to its larger surface area (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 (Talbott 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 (BM) 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 (*U. arctos horribilis*), porosity can even be 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. Still, bears may lose some bone during hibernation but it could be rapidly recovered with remobilization after arousal (Donahue et al., 2003ab, 2006b). Usually, a remobilization period 2–3 times longer than the duration of disuse is required for the full recovery of lost bone, 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 the inactivity. One

potential mechanism is calcium recycling via parathyroid hormone (Donahue et al., 2006a; McGee-Lawrence et al., 2009ab). 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 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.

Recently, 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 BM (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, 2007ab, 2012). With similarities to bears, raccoon dogs display seasonal lethargy (henceforth hibernation) with prolonged catabolism and reduced locomotion, moderately suppressed metabolic rate and slightly decreased body temperature (Mustonen et al., 2007a, 2012). Their BM decreases by approximately 40% during hibernation, which can last up to 9 weeks in northern latitudes (Mustonen et al., 2007a). The appetite decreases during wintering also 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 to 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 also 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 (Council of the European Communities, 1986; http://ec.europa.eu/food/fs/aw/aw_legislation/scientific/86-609-eec_en.pdf) and complied with the ARRIVE guidelines (Kilkenny et al., 2010). Twelve 7-month-old farm-bred raccoon dogs (eleven females and one male) were randomly divided into two groups (n = 6/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 BM of all animals was 13.95 ± 0.41 kg on December 7, 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 fasted for 10 weeks between December 22, 2009 and March 1, 2010. The fasted animals were housed in a separate shadow house to minimize stress during feeding time. All animals received water/ice *ad libitum* and the control group was fasted overnight before the blood samplings. The staff of the Research Farm monitored the health of the animals regularly.

Blood sampling and peripheral quantitative computed tomography (pQCT)

On December 7, 2009, all the raccoon dogs were transported to the Institute of Biomedicine, University of Oulu, Finland. They were anaesthetized with intramuscular ketamine (5 mg/kg) and xylazine (2 mg/kg), weighed and blood samples were collected from a superficial vein of a hind leg into ethylenediaminetetraacetic acid 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 hind limb 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 µm × 590 µm × 1500 µm was measured 2.5 cm proximal from 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 am–noon by researchers unaware of the group assignment. The blood sampling and pQCT measurements were repeated by using the same procedures at the University of Oulu after 9 weeks of food deprivation on February 23, 2010.

Tissue collection

At the end of the 10-week fast, the animals were sacrificed with an electric shock according to the regulations of the Council of the European Union (1993; http://ec.europa.eu/food/fs/aw/aw_legislation/slaughter/93-119-ec_en.pdf). They were weighed, and the right hind limb was removed as a whole and preserved within the surrounding tissues in diluted PBS buffer (Dulbecco's Phosphate Buffered Saline 10×, Sigma-Aldrich, St. Louis, MO, USA) at –80°C for the further analyses.

Micro-computed tomography (µCT)

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 µm isotropic voxel size. The X-ray tube voltage was set to 80 kV, current to 300 µA, and copper–aluminium filter was used to reduce beam hardening. Projection images were collected at every 0.3° over full rotation (360°) and frame averaging of 2 was used. The projections were reconstructed with the NRecon software and standard morphometric parameters (Bouxsein et al., 2010) were calculated using the CTAn software. Both softwares were provided by Bruker microCT. Two volumes of interest (VOI) were manually drawn into the distal trabecular bone and the mid-diaphyseal cortical bone. Both VOI were 17 mm (1000 slices) long and started either 35 mm (2000 slices) or 1.7 mm (100 images) proximal from the distal tibial growth plate.

Biomechanical testing

After µCT imaging, the tibiae were subjected to three-point bending test by placing them on a metal supporter with the fixed span length of 85 mm and loading them until fracture at single point in mid-shaft with the constant loading speed of 0.155 mm/s 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 the HYPERION 3000 FTIRI microscope (Bruker Optics Inc, Billerica, MA, USA) using 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 (FPA). Spatial and spectral resolutions were set to 20 µm and 8 cm^{-1}, respectively. Each spectrum between 800–3300 cm^{-1} was averaged 32 times and two spectral maps of 32 × 32 with a pixel size of 3.9 µm were collected from the anterior and posterior quarters of the bone section. Initial data analyses were conducted with the CytoSpec software (CytoSpec, 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 1585–1720 cm^{-1}, 900–1200 cm^{-1} and 850–890 cm^{-1} (Boskey and Pleshko Camacho, 2007). These maps were subsequently transferred to the 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 the radioimmunoassay

kits of Orion Diagnostica (Espoo, Finland). These analyses were validated for the raccoon dog with dilution series. The alanine (ALT) and 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 the 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. Nonparametric tests were selected due to the relatively small sample size. The p value <0.05 was considered statistically significant. The results are presented as the mean ± SE.

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 ± 0.92% of their BM during the experiment (December 7–March 1), while the decrease in the fasted animals was 31.9 ± 0.77% (Mann–Whitney U test, p=0.004 between the treatments). The pQCT measurements did not reveal any differences between the fed and fasted animals after 9 weeks of fasting (Table 1). Neither did the three-point bending test conducted after 10 weeks reveal differences between the experimental groups (Table 2). In the µCT analysis (Tables 3–4, Fig. 1), the specific bone surface was higher in the fasted raccoon dogs in the trabecular bone of 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 µCT 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 the ICTP values between December and February with higher concentrations after 9 weeks of fasting compared to 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 the Vit D-25 concentrations from December to February. The ALT, AST and AP activities and IP concentrations were unaffected by food deprivation. The seasonal trends in the plasma variables of the fed animals were nonsignificant.

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, µCT 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; Kitao et al., 2009; Mustonen et al., 2012). 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 due to reduced skeletal loading should lead to progressive bone loss in raccoon dogs similar to 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 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 compared to formation (Donahue et al., 2003ab, 2006b). This should eventually lead to net bone loss that is, however, usually not 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. On the other hand, high resolution µCT, 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 would 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 if 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.

Several hormones participate in bone remodeling and they could possibly play roles in the preservation of bone mass in the raccoon dogs. High concentrations of glucocorticoids and thyroid hormones have deleterious effects on bone by inducing resorption and bone loss (Manelli and Giustina, 2000; Gorka 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, on the other hand, 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 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 antiosteogenic 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 (Elefteriou et al., 2004; He et al., 2013). It is unclear whether the persisting leptin concentrations would play 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. 2003ab) which could as a single finding indicate bone loss. Interestingly, the levels of sclerostin that decrease 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 nonsignificant, 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 the ones

subjected to food deprivation (Nieminen et al., 2002). A 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 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 on 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 a 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 the plasma transaminase activities, the 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 BM loss confirmed that the animals remained in phase II of fasting with fat mobilization (Mustonen et al., 2004). It can be outlined 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, µCT, FTIRI and plasma biochemistry, the preservation of bone in wintering raccoon dogs shares similarities to bears. 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 those related to bone formation remained stable. This unbalance 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

No competing interests declared

Author contributions

P.N., A.-M.M. and M.F. designed the study. P.N, A.-M.M., M.F., S.S. and K.P. conducted the laboratory analyses and P.N. performed the statistical analyses. All authors participated in the interpretation of the results. A.-M.M. wrote the first draft of the manuscript. All authors commented on the manuscript and accepted its final content.

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List of Abbreviations

ALT = alanine aminotransferase, ANOVA = analysis of variance, AP = alkaline phosphatase, AST = aspartate aminotransferase, ATR = attenuated total reflection, BM = body mass, BMD = bone mineral density, FPA = focal plane array, FTIRI = Fourier transform infrared imaging, ICTP = C-terminal telopeptide of type I collagen, IP = inorganic phosphorus, μCT = micro-computed tomography, PBS = phosphate buffered saline, PINP = N-terminal propeptide of type I procollagen, pQCT = peripheral quantitative computed tomography, Vit D-25 = 25-hydroxyvitamin D, VOI = volume of interest

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Figures

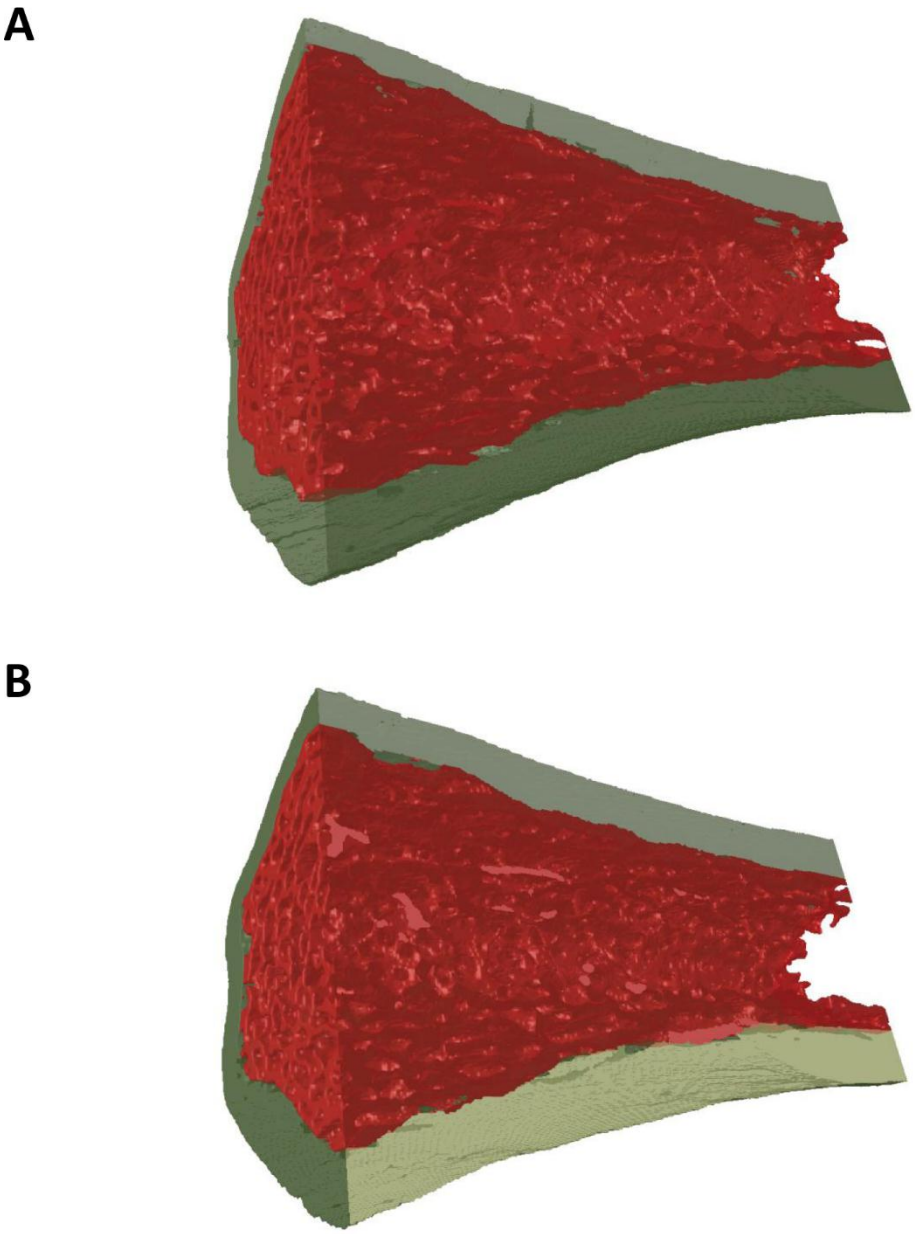

Fig. 1. 3D visualization of cortical (grey) and trabecular (red) bone in the distal tibia. The structure is similar between a fed (A) and a fasted (B) raccoon dog in March after 10 weeks of feeding or fasting.

Tables

Table 1. Osteological peripheral quantitative computed tomography characteristics of the distal tibia of the fed and fasted raccoon dogs before (December) and after (February) the 9-week food deprivation of the fasted group, mean ± SE. No statistically significant differences between the groups or between the time points.

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

Table 2. Variables related to bone strength of the distal tibia of the raccoon dogs in March after 10 weeks of feeding or fasting, mean ± SE. No statistically significant differences between the groups.

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

D = deformation, F = force, E = energy at yield and maximum load.

Table 3. Results of 2D micro-computed tomography analysis in the cortical bone of mid-diaphyseal tibia of the raccoon dogs in March after 10 weeks of feeding or fasting, mean ± SE. No statistically significant differences between the groups.

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

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

	Fed	Fasted
Bone volume (mm^3)	426 ± 43.3	370 ± 17.8
Trabecular bone fraction (%)	26.4 ± 1.56	22.9 ± 1.52
Bone surface (mm^2)	8488 ± 762.0	8328 ± 488.1
Specific bone surface (mm^{-1})	20.1 ± 0.31*	22.5 ± 0.48*
Bone surface density (mm^{-1})	5.3 ± 0.27	5.2 ± 0.36
Trabecular thickness (mm)	0.17 ± 0.002*	0.15 ± 0.002*
Trabecular separation (mm)	0.55 ± 0.037	0.64 ± 0.138
Trabecular number (mm^{-1})	1.59 ± 0.087	1.51 ± 0.107
Trabecular pattern factor (mm^{-1})	–0.13 ± 0.794	0.78 ± 0.474
Structure model index (AU)	0.61 ± 0.141	0.80 ± 0.092
Degree of anisotropy (AU)	2.09 ± 0.083	2.12 ± 0.035
Fractal dimension (AU)	2.52 ± 0.016*	2.47 ± 0.010*
Euler number (AU)	–18716 ± 2710.2	–19453 ± 2949.6
Connectivity (AU)	28353 ± 3446.9	30166 ± 2664.8
Connectivity density (mm^{-3})	17.5 ± 1.45	18.7± 1.79

AU = arbitrary unit

* = Statistically significant differences between experimental groups (Mann–Whitney U test, $p<0.05$).

Table 5. Fourier transform infrared imaging results of the distal tibia of the raccoon dogs in March after 10 weeks of feeding or fasting, mean ± SE. No statistically significant differences between the groups.

	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

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

	Dec		Feb	
	Fed	Fasted	Fed	Fasted
PINP (µg/l)	2.6 ± 0.2	2.4 ± 0.3	2.1 ± 0.4	2.2 ± 0.4
ICTP (µg/l)	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)	2.9 ± 0.8	2.8 ± 0.6	2.3 ± 0.4	4.2 ± 1.2
ALT (U/l)	62 ± 8	61 ± 9	60 ± 6	40 ± 8
AST (U/l)	50 ± 4	50 ± 6	64 ± 10	48 ± 5
Vit D-25 (nmol/l)	188 ± 12	199 ± 16	148 ± 9	105 ± 8$^{\#}$
IP (mmol/l)	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.

*= Differs statistically significantly from the Fed group at the same time point (Kruskal–Wallis ANOVA, $p<0.05$).

$^{\#}$ = Differs statistically significantly from the previous values of the same experimental group (Kruskal–Wallis ANOVA, $p<0.05$).