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BONE ASSESSMENT OF FREE-LIVING RED SQUIRRELS (*SCIURUS VULGARIS*) FROM THE UNITED KINGDOM

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ABSTRACT: Metabolic bone disease has been reported in free-living red squirrels (*Sciurus vulgaris*) in the United Kingdom but the prevalence of this disease is unknown. In this study the bone quality of free-living red squirrels in the UK was assessed by radiology and bone densitometry. The study comprised 20 red squirrels found dead and submitted to the Zoological Society of London (UK) between 1997 and 1998, 10 were from the Isle of Wight (IoW), where gray squirrels (*Sciurus carolinensis*) are absent, and 10 were from Cumbria (Cu), where gray squirrels are present. Gray squirrels are considered potential competitors for red squirrels. Radiologic evaluation of humerus, femur, tibia, radius, and ilium revealed a slightly lower bone density and thinner cortices in red squirrels from the IoW when compared with those from Cu. Dual-energy X-ray absorptiometry was used to measure bone mineral content and density of the isolated right humerus and femur of 19 of the 20 red squirrels. The bone densitometry study reinforced the radiographic findings. The IoW specimens had lower bone mineral density values, although statistical significance ($P<0.05$) between animals from the IoW and Cu was only reached for the proximal epiphysis of the femur and between males from the IoW and males from Cu for the proximal epiphysis of the humerus. A highly positive correlation ($r>0.94$) was found when the bone mineral content and density between the femur and the humerus among groups and within each group were compared, showing a uniform level of mineralization between upper and lower limbs. These findings suggested generalized bone loss for the IoW red squirrels that may be compatible with some degree of osteopenia. Within the wide range of causes that lead to osteopenia, malnutrition (especially protein deficiency), calcium and copper deficiencies, and genetic factors remain as possible etiologies.

Key words: Bone, densitometry, gray squirrel, radiology, red squirrel.

INTRODUCTION

Red squirrels (*Sciurus vulgaris*) are part of the wild fauna of the UK, where they fill an important ecologic niche (Gurnell and Pepper, 1991). Red squirrel population numbers have fluctuated widely over the last 300 yr, perhaps because of a dependence on tree seed availability (Gurnell and Pepper, 1993). The gray squirrel (*Sciurus carolinensis*) was introduced into the UK from North America in the 1800s (Gurnell and Pepper, 1993). Red squirrels disappeared from most British deciduous and mixed woodland in the 15- to 20-yr period after arrival of gray squirrels in these areas (Gurnell, 1993). Decline of red squirrels in the British Isles and replacement by gray squirrels continues. Red squirrels may become extinct from mainland England and Wales, where populations are already low and fragmented, within the next 10–20 yr unless measures to conserve them are established (Gurnell and Pepper, 1991). Reasons for red squirrel population declines are uncertain and possibly due to a combination of factors (Gurnell and Pepper, 1993; Kenward and Holm, 1993). Habitat specialization, social interactions with gray squirrels, competition for food with gray squirrels, lower efficacy of food utilization than gray squirrels, and parapoxvirus disease epidemics are some factors that may play a role in their population decline (Gurnell and Pepper, 1991, 1993; Gurnell, 1993; Sainsbury and Gurnell, 1995, 1997; Sainsbury and Ward, 1996; Kenward and Hodder, 1998; Kenward et al., 1998).

Metabolic bone disease (MBD) encompasses several syndromes and its occurrence in captive wild animals is due to inadequate husbandry and dietary mismanagement (Fowler, 1986). A few reports have been made of MBD in free-living wild mammals (Van Pelt and Caley, 1974; Cologue et al., 1979; Bleich et al., 1990; Hindelang and Peterson, 1996; Ytrehus et al., 1999). Metabolic bone disease in red squirrels has not been considered a threat in conservation programs (Sainsbury and Gurnell, 1995), despite diagnosis of nutritional bone diseases in wild-caught red squirrels kept in captivity (Rings et al., 1969; Gurnell et al., 1990) and in two free-living red squirrels (Keymer and Hime, 1977). The diet of red squirrels in the wild includes Scot pine seeds and fungi as a main source of food, but they also eat fruits, berries, buds, shoots, flowers, bark, invertebrates, and lichen, and it is known that squirrels routinely chew animal bones found in their environment (Allan, 1935; Carlson, 1940; Coventry, 1940). Analysis of the main food sources showed low calcium content and low calcium to phosphorus ratios (Gurnell et al., 1990). Therefore, red squirrels may have predisposition to metabolic bone disease (Sainsbury and Gurnell, 1995), and if gray squirrels exert competitive exclusion on red squirrels (Gurnell and Pepper, 1993), this could enhance their predisposition to bone disorders.

Radiology has been used as a key tool to diagnose bone disorders. However, metabolic bone lesions that can be seen radiologically do not occur until late in the course of the disease (Fowler, 1986). Dual-energy X-ray absorptiometry (DEXA) is a radiographic technique based on exponential attenuation; it measures bone mass and is used to detect early bone loss and can indicate risk of fracture and osteoporosis. Although trabecular bone is usually much more metabolically active than cortical bone and hence ought to be more sensitive to changes in bone density, trabecular bone loss is difficult to detect with plain radiographs (Mayo-Smith and Rosenthal, 1991) and radiographic techniques such as DEXA can be used to increase the sensitivity to early bone loss.

Assuming that the red squirrel population decline in the UK is a consequence of a combination of causes, a multidisciplinary investigation is required. This study is part of a larger study on causes of morbidity and mortality in red squirrels and the effect of gray squirrels on red squirrel populations. The aim of this preliminary study was to test the hypothesis that bone mass loss in red squirrels is related to competition with gray squirrels for food. The study included examination of bones of a subset of the population of free-living red squirrels in the UK by using plain radiography and DEXA. Red squirrels chosen for the study were from a population where gray squirrels are present (Cumbria [Cu], UK) and from one where gray squirrels are absent (Isle of Wight [IoW], UK).

MATERIALS AND METHODS

Origin of samples

Red squirrels found dead throughout the UK were submitted to the Institute of Zoology, Zoological Society of London (London, UK) for a survey of causes of mortality. Deaths of 10 red squirrels from the IoW and 10 from Cu between 1997 and 1998 were associated with trauma (probable road traffic accident). The 20 animals were in good body condition (four were scored as fat and 16 were scored as normal body condition) and therefore were assumed to be healthy at the time of death. They were frozen and thawed several times before and during the study. The red squirrels were defined as subadults if their bony growth epiphyseal plates had not closed and adults if the epiphyseal plates had closed and the animal was more than 200 g body weight.

The Cu group comprised seven males (M) and three females (F), of which two were subadults (two M) and eight were adults and had a mean body weight of 299±51.8 g (range 220–385 g). The IoW group comprised four M and six F, of which three were subadults (two F and one M) and seven were adults with a mean weight of 305.5±44.5 g (range 240–365 g).

Radiology

Whole-body ventrodorsal radiographs, with extended limbs taking care to avoid superimposition of the bones and excluding the tail, were taken of all animals with the same settings. A Dean D.38 Mobile Unit with a Dynamax 40 dual-focus tube capable of delivering 75–125 kV and 25–300 mA (Dean, Croydon, UK) was used. Radiographs were made on Fujifilm UM-MA HC film (Fuji PhotoFilm Ltd., London, NW3, UK), mammography screen type, at 91 cm focus film distance, with an exposure time of 0.16 sec, at 50 mA and 82 kV. The films were wet processed in a Polycon X-ray developer (May and Baker, Essex, UK) at 20 C.

Because of the small size of the skeleton of red squirrels, only long bones (humerus, radius, femur, and tibia) and ilium were assessed radiologically, by using a magnifier loupe. The bones were assessed and scored for cortical erosion, both subperiosteal (roughening of the subperiosteal surface and loss of the cortical outline; 0 = no erosion; 1 = slight erosion; 2 = severe erosion) and endosteal (scalloped appearance of the inner cortex; 0 = no erosion; 1 = slight erosion; 2 = severe erosion), and coarseness of trabeculae (trabecular definition and coarseness; 0 = good trabecular definition; 1 = poor and unsharp definition; 2 = severe coarseness of the trabeculae). Cortical breadth was measured by using the technique of Barnett and Nordin (1960) as follows. The diaphysial length of the bone was measured with a ruler and a line was drawn across the midline of the diaphysis at 90 degrees to the long axis of the bone. A graticule with a 2× magnifier lens was then used to measure the breadth of both cortices (BC1 and BC2) in millimeters and the total breadth of the bone (BB). Then the cortical bone index (CBI) was calculated by using the following equation: $100\times(BC1+BC2)/BB=CBI$. This was expressed as a percent of the breadth of the bone; BC1+BC2 is the breadth of mineralized bone in millimeters.

Isolated bones (right humerus and femur) from each of 19 red squirrels, nine from the IoW and 10 from Cu, were scanned on a QDR-1000/W densitometer (Hologic, Waltham, Massachussets, USA) calibrated with a Hologic hydroxyapatite anthropomorphic spine phantom. An ultrahigh-resolution software program (provided by the manufacturer), which increased the resolving powder (line spacing was set at 0.0254 cm and resolution at 0.0127 cm), was used to calculate the mineralized bone area, bone mineral content (BMC), and the bone mineral density (BMD). The bones were immersed in a water bath at a depth of 1 cm and were placed in cranial–caudal position. The scan area for the humerus and femur was length 4.978 and 5.994 cm and width 1.905 and 2.016 cm, respectively. Time required was 10.52 min for each scan of the humerus and 13.01 min for the femur. Scans were carried out on 2 days by the same researcher (R.M.G.). One humerus was scanned six times on the same day, with repositioning between scans. Reproducibility was evaluated by calculating the coefficient of variation ($CV=100\times SD/mean$; where SD is standard deviation). Bone mineral content (g) and BMD (g/cm^2) were calculated from the entire humerus and the entire femur as a global bone and by partitioning the femur and the humerus into proximal epiphysis (subregion R1), diaphysis (R2), and trabecular bone regions at the middiaphysis, excluding cortical bone (R4), in order to assess possible differences in trabecular and cortical proportions. To assess the precision of DEXA measurements performed in red squirrels, the CV was calculated by measuring one isolated humerus six consecutive times with repositioning.

Statistical analysis

Statistical analysis was performed using GraphPad Instat software (Curriculumonline, Bath, BA1 7DD, UK). A Student's *t*-test with unpaired data and two-sided *P* value was used to calculate the mean difference between two populations (IoW and Cu) by comparing the variables sex, weight, age, and BMD.

RESULTS

None of the animals showed radiographically detectable cortical erosions or trabecular coarseness. Five of the animals, four from the IoW (two F subadults, one F adult, and one M subadult) and one from Cu (F adult) showed slightly low radiologic bone density compared with the other animals radiographed.

Cortical bone index means, SDs, and ranges are presented in Table 1. Table 2 shows the statistical results of the comparison between the means of the CBI. A clear difference in cortical breadth was found between the two groups; the IoW specimens had thinner cortices and the difference was statistically significant for the CBI of the humerus and the tibia.

The five red squirrels that were found to have lower bone density on a radiograph also had the lowest BMD measurements. Two other females that were pregnant, one from Cu and the other from the IoW, had BMDs in the highest range of values (Table 3).

Specimens from Cu had higher BMDs compared to IoW specimens for subregion R1 of the femur (Cu: 0.1653±0.0144 vs.

TABLE 1. Mean, standard deviation (SD), and range of cortical bone index (CBI) in red squirrels from the Isle of Wight (IoW) and Cumbria (Cu).

	CBI	Femur	Tibia	Radius	Humerus
IoW	Mean	29.64	36.75	43.21	27.83
	SD	3.53	4.31	6.50	4.04
	Range	23.52–35.29	30.30–43.75	33.30–55.00	22.22–34.37
Cu	Mean	30.34	41.69	44.81	32.61
	SD	2.92	5.97	4.05	3.13
	Range	26.47–36.36	32.25–55.88	40.00–54.16	28.12–37.5

IoW: 0.1457±0.0163, $P< 0.05$). The BMD of the global femur for Cu squirrels was higher than the BMD of IoW squirrels but the difference only approached significance (Cu: 0.1911±0.0129 vs. IoW; 0.1754±0.0203, $0.05<P<0.1$) For the humerus, when total populations were compared, Cu squirrels had a higher BMD for subregion R1 but the difference only approached significance (0.2015±0.013 vs. 0.1876±0.0187; $0.05<P<0.1$). When values of males from both populations were compared, subregion R1 of the humerus of Cu squirrels had higher values with a significant mean difference (Cu: 0.2004±0.0111 vs. IoW: 0.1818±0.0086; $P<0.05$). A high correlation ($r>0.94$) was found between the femur and humerus BMC and BMD for each population. The CV of the isolated humerus measured six consecutive times with repositioning was 1.14%, indicating good precision of DEXA measurements in the bones of red squirrels.

DISCUSSION

Bone is a dynamic tissue that is in constant change (remodeling) due to two physiologic processes, resorption (by osteoclast cells) and formation (by osteoblast cells) of the bone. Structure and morphology of bone are determined by genetic and environmental factors. Radiology is often used for initial evaluation of the skeletal system. It is available, inexpensive, and reliable. Radiology has been used as an essential tool to diagnose MBD. However, MBD lesions that can be seen in radiographs do not occur until late in the course of the disease (Fowler, 1986). Harris and Heaney (1969, cited by Mayo-Smith and Rosenthal, 1991) stated that 30–50% of skeletal calcium must be lost before abnormalities can be identified radiographically.

Bone loss in adults can occur slowly or rapidly depending on the cause (Mayo-Smith and Rosenthal, 1991). Characteristically in slow bone loss, remodeling results in a thin cortex due to endosteal resorption. Cortical thinning is a useful guide for the presence of osteopenia. In slow bone loss, the non–weight-bearing trabeculae are resorbed first, leading to a prominence and thickening of the residual trabeculae. Findings associated with rapid bone loss do not reflect mechanical needs, but rather the mechanism of resorption. Radiographically, changes associated with rapid bone loss are intracortical tunneling (lucencies parallel to the long axis of the

TABLE 2. Statistical comparison between the means of cortical bone index (CBI) of the right femur, tibia, radius, and humerus from Cumbria (Cu) and Isle of Wight (IoW) red squirrel populations. Underline values indicate statiscal significance. The mean difference is the difference between the IoW, CBI, and the Cu CBI means.

	Femur	Tibia	Radius	Humerus
Mean difference	0.70	4.95	1.60	4.78
P-value	0.63	<u>0.05</u>	0.53	<u>0.01</u>

TABLE 3. Results of assessment of red squirrel bones by radiology and dual-energy X-ray absorptiometry.

Reference no.	Sex[a]	Weight (g)	Origin[b]	Age[c]	G-H-BMD[d] (g/cm^2)	G-F-BMD[d] (g/cm^2)	R1-H-BMD[d] (g/cm^2)	R1-F-BMD[d] (g/cm^2)	R2-H-BMD[d] (g/cm^2)	R2-F-BMD[d] (g/cm^2)	R4-H-BMD[d] (g/cm^2)	R4-F-BMD[d] (g/cm^2)
424-98[e]	F	240	IoW	SA	0.1514	0.1499	0.1726	0.1346	0.1455	0.1404	0.1453	0.1257
545-98[e]	F	365	IoW	A	0.1744	0.1694	0.1573	0.128	0.1908	0.178	0.1964	0.141
555-98	F	320	IoW	A	0.1811	0.1846	0.1896	0.1463	0.1911	0.1897	0.1919	0.1686
557-98[e]	M	280	IoW	SA	0.1555	0.157	0.1848	0.1433	0.1598	0.1492	0.154	0.1422
632-98	F (P)	335	IoW	A	0.2041	0.2027	0.2077	0.1697	0.2153	0.2071	0.2288	0.1876
673-98	F	345	IoW	A	0.2052	0.2009	0.2178	0.1747	0.2141	0.2014	0.2318	0.179
819-97	M	295	IoW	A	0.1979	0.188	0.1886	0.1572	0.2077	0.1865	0.2193	0.168
802-97[e]	F	235	IoW	SA	0.1578	0.15	0.1981	0.133	0.1494	0.1408	0.1387	0.1129
035-97	M	345	IoW	A	0.175	0.1758	0.1721	0.1427	0.189	0.1765	0.1924	0.1467
680-98	M	350	Cu	A	0.2158	0.2179	0.1954	0.1897	0.2424	0.2216	0.2679	0.2004
658-98	M	285	Cu	A	0.1837	0.1896	0.1817	0.1536	0.198	0.194	0.2026	0.1654
613-98	M	245	Cu	SA	0.1747	0.1837	0.215	0.1605	0.1734	0.1753	0.1751	0.1476
597-98	M	275	Cu	SA	0.1675	0.1804	0.2002	0.1589	0.1667	0.1717	0.1708	0.149
587-98	F	325	Cu	A	0.1819	0.1928	0.1912	0.1566	0.1923	0.1973	0.1879	0.1637
585-98	M	300	Cu	A	0.1888	0.1957	0.2054	0.1629	0.2012	0.1997	0.1966	0.1749
575-98[e]	F	220	Cu	A	0.174	0.1768	0.1946	0.1485	0.1843	0.1758	0.2075	0.1748
573-98	M	260	Cu	A	0.1682	0.1756	0.195	0.1565	0.1729	0.1708	0.18	0.1585
505-98	M	385	Cu	A	0.1989	0.2023	0.2104	0.1814	0.2093	0.2008	0.2412	0.1853
487-98	F (P)	345	Cu	A	0.1996	0.1959	0.2265	0.1847	0.2048	0.1879	0.2245	0.1661

[a] F = female; M = male; P = pregnant.
[b] IoW = Isle of Wight; Cu = Cumbria.
[c] SA = subadult; A = adult.
[d] G = global; H = humerus; BMD = bone mineral density; F = femur; R1 = epiphysis proximal; R2 = diaphysis; R4 = trabecular bone.
[e] Low bone radiographic density.

bone), poor definition of cortical surfaces mainly due to subperiosteal resorption, and coarseness of the trabecular bone. In general, these features are related to disease states with rapid turnover of bone such as hyperparathyroidism (Hayes and Conway, 1991).

A clear difference in cortical thickness of bones was found between the Cu and IoW squirrels. The IoW red squirrels had thinner cortices, particularly evident in comparison of the CBI of the humerus and the tibia. Because of a lack of reference data for squirrel bones, it is not possible to confirm whether the values found are in a normal range. Thin but well-defined cortices and low bone density seen in the red squirrels in our study are most likely to have been caused by slow bone loss. Although, we did not find radiologic evidence of skeletal deformity suggesting osteomalacia or rickets, further histologic study of the bones would be necessary to establish coexistence of a mineralization defect.

Differences in the global femur BMD and in subregions R1 of the femur and the humerus were found on the DEXA scans. The Cu squirrels had the highest values of BMD in the global femur and in subregions R1 in both femur and humerus; R1 corresponds to the proximal epiphysis of the femur and the humerus and measures predominantly trabecular bone rather than cortical bone. The difference seen between the Cu and IoW squirrels correlates with the difference when comparing males of the two groups, showing a significant difference in the R1 for the humerus ($P<0.05$) and an approached significance for the R1 of the femur ($P<0.1$). In human medicine, the proximal epiphyses of the femur and humerus are considered the most common sites of reduction of bone density when osteopenia develops (Gillespy and Gillespy, 1991). High positive correlations ($r>0.94$) were found when comparing BMC and BMD between the femur and humerus among both groups and within each group, showing a uniform pattern of mineralization between upper and lower limbs and suggesting a generalized low bone mass for the IoW red squirrels. Factors intrinsic to the IoW group could be the reason for low bone density, including genetic factors, low bone mass at birth, or to failure to achieve an optimum bone density during growth.

Dual-energy X-ray absorptiometry has proved an accurate and precise technique to detect bone loss when used on rats (Griffin et al., 1993; Bruce et al., 1994; Ladizesky et al., 1994; Mitlak et al., 1994; Gala Paniagua et al., 1998). The coefficient of variation was 1.14%, showing good precision of the DEXA scans when measuring squirrel bones. To measure the accuracy of the technique it would be necessary to calculate the ash weights of the bones measured and correlate them with the DEXA measurements. Studies in rats indicated a high positive correlation between ash weights and BMC. Suttie et al. (1983) performed regression equations relating mineral content of the metacarpus of red deer (*Cervus elaphus*) to radiographic cortical breadth measurements. They found that the best correlations existed between calcium and phosphorus content and breadth of mineralized bone. However, correlation of CBI with mineral status was poor, which might have been because the metacarpus was used rather than the femur.

Thin cortices and low bone density suggested that some degree of osteoporosis existed in the IoW squirrels. Causes of osteoporosis include malnutrition (especially protein and calcium deficiency), estrogen deficiency (human postmenopausal), hyperadrenocorticism, hyperpituitarism, and hyperthyroidism. Also, vitamin C deficiency and copper deficiency result in osteoporosis. Other techniques of analysis such as microradiography and histology would be necessary to evaluate the changes in the bones to determine if they were normal or abnormal. Hyperadrenocorticism, hyperpituitarism, and hyperthyroidism are unlikely in the IoW group because the squirrels apparently were healthy. Estrogen deficiency can be discounted because the effect was seen also in male squirrels. Rodents, except guinea pigs, do not require vitamin C in their diet. Protein, calcium, and copper deficiencies could be responsible for the possible osteoporosis because of lower protein in the diet or lack of access to bones. It is also possible that differences in the bones of the groups are due to the small numbers of animals studied or some other bias.

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