Influence of the calcium content of the diet offered to leopard tortoises (*Geochelone pardalis*)

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Twenty-four juvenile leopard tortoises were divided into four groups of six; one group was fed a basic low-calcium feed for six months, and the other three groups were fed the same basic diet supplemented with one, three and nine times the amount of calcium recommended as a supplement to the diet of reptiles. The animals' bone mineral content and bone mineral density were estimated by dual energy x-ray absorptiometry, and blood samples were taken at the start and at the conclusion of the study. One tortoise from each group was examined postmortem. There was a clear depletion of calcium in the body of the tortoises receiving no calcium supplement, and the shell of the tortoises receiving the recommended calcium supplement did not calcify to the extent expected. The tortoises that received three times the recommended calcium supplementation had the highest growth rate and were thriving. However, metastatic calcifications were observed postmortem in the two groups that were given the highest doses of calcium.

THE leopard tortoise (*Geochelone pardalis*) is primarily herbivorous. Milton (1992) described the natural diet of the species as consisting of succulents, forbs, grasses, shrubs, fruit and occasionally insects, small stones and bone fragments. According to Jacobson (1989), captive herbivorous reptiles are especially prone to nutritional deficiencies when their food lacks minerals, and they are particularly exposed to calcium deficiency because their only source of calcium may be the cultivated vegetables offered to them, some of which may be very low in calcium. As a result, mineral deficiencies are more common in herbivores than in carnivores, probably because carnivores have access to a more diverse selection of minerals in their diet.

In reptilian medicine, most of the pathological processes encountered are related to inadequate husbandry and management. This may not be apparent to many small animal clinicians because factors such as temperature, humidity, light cycles and exposure to natural sunlight rarely make a difference to the health of a dog or a cat (Mader 2000). In the USA, it is estimated that nutritional diseases constitute approximately 40 per cent of the causes of death of reptiles in captivity, and that their importance is often underestimated. Reptile nutrition is complex because the requirements of the 6400 species of reptile vary widely, and the metabolism and nutritional requirements of each species are closely related to their environment (Schilliger 2000). Most nutritional problems become apparent during the animals' growth, and disorders related to calcium metabolism are probably the most important in the management of captive reptiles (Scott 1992). Wallach (1977) pointed out that calcium deficiency is the most common nutritional disease encountered in captive reptiles, although in the wild nutritional deficiencies are rarely seen. Tortoises, for example, apparently regulate their need for nutrients by eating a wide variety of plant species and parts. In northern Tanzania, the leopard tortoise was observed to eat 47 different plant species (Kabigumila 2001). Tortoises have also been reported to ingest bones, stones and soil, which can all be sources of calcium, and it may be that it is this mineral that the tortoises seek. Research is required to determine whether the vegetable diet of wild tortoises is deficient in nutrients or minerals, as the ingestion of stones, soil and bones is more common than previously assumed (Esque and Peters 1994).

Metabolic bone disease is the most common medical disorder in captive chelonians and is often nutritional in origin. It occurs most commonly in growing juveniles and in reproductively active females, in which skeletal modifications are occurring (Frye 1991), and it includes a number of conditions that develop as a result of prolonged deficiencies of calcium and vitamin D or an unsatisfactory ratio of calcium to phosphorus in the diet. It should be considered as a disease caused by dietary and husbandry mismanagement, characterised by metabolic defects that affect the morphology and function of the bones. Its clinical, radiographical and pathological signs may vary with the age of the animal, the degree and duration of the deficiency, and the presence of concurrent diseases (Fowler 1978). The affected animal will grow slowly and be undersized for its age. If the young chelonian is maintained on an inadequate diet, the shell may fail to calcify and remain soft, rather than becoming firm and unyielding (Redrobe 1996). Calcium has a number of essential functions in the body, but in terms of the quantity of calcium required, its predominant role is to provide structural stability as the major cation of crystalline hydroxyapatite in bone. In bone, calcium and phosphorus are almost always present in a ratio of 2:1 (Phillips 1977). Many authors recommend a dietary calcium:phosphorus ratio of 1·5 to 2·0:1 for reptiles, the higher calcium levels being required by juveniles and breeding females. On the basis of this recommendation most vegetable diets fed to captive reptiles need some supplementation, which should be in the form of calcium without phosphorus (Scott 1992). However, there is considerable disagreement about the amount of calcium that should be added to the diet of captive tortoises. The aim of this study was to evaluate the effects of calcium supplementation in the diet of tortoises that live in terraria all year round and have little or no access to sunlight.

MATERIALS AND METHODS

Animals and housing

Twenty-four juvenile leopard tortoises (Fig 1) were housed in the Danish Reptile Zoo. Each tortoise was marked with a spot placed directly on its carapace; 14 of them were six months old and 10 were 18 months old. They were divided into two groups of the same weight range to ensure a homogeneous intake of feed. They were then divided into four groups of six tortoises and each group was randomly assigned a specific level of calcium supplementation. The tortoises were placed in four identical indoor terraria with underfloor heating. The terraria they measured 1 m × 1 m, and the floor was covered

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TABLE 1: Quantities of vitamins and minerals added per kg fresh weight of the feed supplied to all the tortoises

Component	Amount
Phosphorus (g)	1·5
Sodium (g)	0·16
Magnesium (g)	0·05
Vitamin A (iu)	3000
Vitamin D_3 (iu)	400
Vitamin E (mg)	5
Vitamin B_1 (mg)	0·3
Vitamin B_2 (mg)	1·2
Vitamin B_6 (mg)	0·1
Vitamin B_{12} (µg)	5
Vitamin K_3 (mg)	0·3
Folic acid (mg)	0·1
Pantothenic acid (mg)	3
Nicotinic acid (mg)	6
Choline chloride (mg)	15
Methionine (mg)	100
Lysine (mg)	200
Zinc (mg)	5
Iron (mg)	3
Manganese (mg)	7
Copper (mg)	0·2
Cobalt (mg)	0·015
Iodine (mg)	0·03

with paper; in one corner, a basking site was provided by placing a tile measuring 25 cm x 25 cm; the opposite corner was used for food and water bowls. The terraria were lit for 10 to 12 hours a day by new HQL UV 80 W light bulbs (Osram) emitting UV-A and UV-B rays, to provide the recommended photoperiod for tortoises in captivity (Beynon and Cooper 1991, Frye 1995). The air temperature was maintained at an optimal average of 25°C, and the temperature at the basking site was approximately 50°C. The relative humidity, measured continuously with a hygrometer, was approximately 70 per cent.

Diet and calcium supplementation

The tortoises were offered a basic diet, relatively poor in calcium, consisting of a mixture of chopped vegetables such as carrots, iceberg lettuce, cucumber, sweet peppers, tomatoes and hay. No ingredients known to be rich in protein, such as alfalfa, were included in the diet. Each day, each group was given 155 g of this fodder. The average calcium content of the daily feed ration for each group was estimated to be approximately 60 mg over the whole experimental period (Moeller 1996). In addition, each group received the same quantity of a supplement containing vitamins, magnesium and sodium (Vitamin and Mineral Mixture for Birds and Reptiles; Diafarm) (Table 1). The diets of groups 2, 3 and 4 were further supplemented with calcium to give the total calcium contents specified in Table 2; the tortoises in group 2 received the calcium supplement (Diafarm) for reptiles (based on calcium carbonate) at the recommended dose of 2·55 g/kg fresh weight of prepared feed, and the tortoises in groups 3 and 4 were provided with pure calcium carbonate to achieve the higher levels of calcium given to these two groups. The appetite of the tortoises was satisfied daily. The intake of fodder was monitored over the whole period by weighing the unconsumed fodder daily. A fresh supply of water was available at all times.

TABLE 2: Total quantities of calcium (g/kg fresh weight of prepared feed) supplied to the four groups of tortoises

Group	Amount
1	0·4
2	2·9
3	8·0
4	23·3

FIG 1: Juvenile leopard tortoise (*Geochelone pardalis*)

Growth

The bodyweight of the tortoises was recorded once a month during the six months of the study. No other measurements of growth were made, but the tortoises were given a general clinical examination once a month, which included an examination of the shell for signs of softness or distortion, including pyramidal doming (Mas 2001).

Bone scanning

Bone mineral content (BMC) and bone mineral density (BMD) were estimated by dual energy x-ray absorptiometry with a QDR-2000 bone densitometer (Hologic), using software designed for the whole-body examination of rats. The tortoises were scanned at the start and at the conclusion of the study; they were immobilised during the scans by fixing their legs and head in a retracted position with tape, ensuring that they had breathing space; the application of the tape did not affect the measurements.

Blood biochemistry

A MSCAN Vet chemistry analyser (Melet Schloesing Laboratoires) was used for the biochemical analysis of tortoise blood, after its precision had been verified against the ADVIA 1650 analyser (Bayer) for a number of blood parameters using blood from five healthy spur-thighed tortoises (*Testudo graeca*). Blood samples were collected from each tortoise at the start and at the conclusion of the study; the preferred method was by puncture of a jugular vein (Murray 2000), but when this was not possible the samples were taken by puncture of a dorsal coccygeal vein (Samour and others 1984); they were taken into a syringe coated with lithium heparin. A minimum of 100 µl whole blood was used for the analysis. The parameters determined were alkaline phosphatase, alanine aminotransferase, total bilirubin, blood urea nitrogen, cholesterol, ionised calcium, albumin, creatinine, glucose, potassium, phosphorus and total proteins.

Postmortem examination

Four tortoises, one from each group, were chosen at random and euthanased by the intravenous injection of an overdose of pentobarbital (Cooper and others 1989); a complete postmortem examination was made of each animal, and samples from the liver, kidneys, intestine, heart, lungs and long bones were fixed in 10 per cent buffered formalin. The tissues were embedded in paraffin, sectioned at 5 µm and stained with

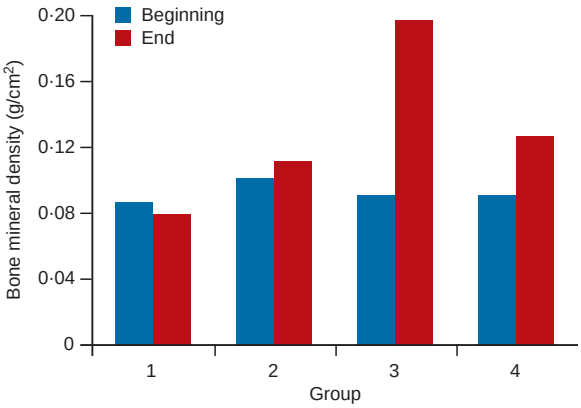

FIG 2: Bone mineral density of the four groups of tortoises at the beginning and end of the experiment, as determined by dual energy x-ray absorptiometry

haematoxylin and eosin and with Von Kossa stain for the demonstration of calcium salts (Slauson and Cooper 1990).

Data analysis

The data were analysed by using the statistical facilities in Microsoft Excel. Statistics were compiled by using only the animals that survived the whole period of the study. After testing for normal distribution, the mean values were compared by using both paired and heteroscedatic two-tailed and one-tailed Student's *t* tests. The results were recorded as significant at $P<0·05$.

RESULTS

Growth and clinical findings

No shell distortion, including pyramidal doming, was seen, and only a softening of the shells was observed. The weight of the tortoises in group 1 increased on average by 7·5 per cent, the lowest rate of growth observed in any of the four groups. Group 2 had the slowest growth rate at the beginning of the study, but the rate gradually increased and over the whole period their weight increased on average by 11·8 per cent. Group 3 consistently had the highest rate of growth, with a total average gain of weight of 14·5 per cent by the end of the study. The growth rate of group 4 increased steadily but its total average weight gain was only 8·1 per cent. The tortoises which received no calcium supplementation (group 1) became weak and lethargic, and their shells were generally soft; one had an extremely soft shell but survived. Unfortunately, three of the six tortoises in group 2 (receiving the calcium intake recommended by Diafarm) died during the study. They were lethargic, had soft shells and oedema, and a black extravasion could be observed on the plastron. The cause of death was ascertained to be a flagellate infection with *Hexamita parva,* which had infected the tortoises before the study. Although they had been treated with metronidazole (Flagyl; May and Baker) and seemed to have recovered, they did not thrive and died within four months of beginning the study. Of the remaining three tortoises in group 2, two had soft shells and one was thriving. The tortoises which received three and nine times the recommended level of calcium supplementation (groups 3 and 4) were all lively and thriving; they had hard and well developed shells.

Scanning

The total bone and shell weights (proportional to their calcium content) were measured by scanning the tortoises at the beginning and end of the experiment (Fig 2). The tortoises in group 2 were slightly more calcified at the start of the experiment than those in the other groups. In groups 1 and 2 there was no statistically significant increase in the level of calcification during the experiment, whereas in groups 3 and 4 there were significant increases.

Blood biochemistry

At the beginning of the experiment the mean concentration of calcium in the tortoises of group 1, 2·65mM, was significantly higher than the range of 2·18 to 2·25mM in the other three groups. However, at the end of the experiment group 1 had a lower blood calcium level (1·82mM) than the average of all the groups (2·07mM). There were increases in the activity of alkaline phosphatase and in the concentration of phosphorus in the tortoises of group 1, but the changes were not statistically significant. There were no apparent changes in these variables in the other three groups. The activity of alanine aminotransferase was slightly higher in groups 1 and 3 at the end of the study. There were no significant changes in total proteins, bilirubin, blood urea nitrogen, cholesterol, albumin, creatinine, glucose, potassium or total proteins in any of the tortoises.

Postmortem examination

The group 1 tortoise had a thin plastron which was easily cut with a pair of scissors. Its heart appeared soft and shapeless. It had cystic dilations of the intrahepatic biliary ducts, and its femurs had thin cortices. Its gastric mucosa was smooth, without folds. The group 2 tortoise also had a thin plastron which could be cut with a pair of scissors. The texture of its inner organs was fragile. The cortices of its femurs were thicker than those of the group 1 tortoise, and calcifications were observed in focal areas of the connective tissue and in its renal tubuli and lungs. Its gastric mucosa was smooth, without folds. The group 3 tortoise had a hard plastron and carapace, and an oscillating saw was required to separate them. There were large deposits of fat in its abdomen, and its liver was swollen and pale. There were large, well-developed folds in its gastric mucosa. There were multifocal calcifications of the connective tissue in the lungs, focal areas of calcification in the heart, steatosis hepatis, and the cortices of its femurs were thick. The group 4 tortoise also had a hard plastron and carapace and an oscillating saw was required to separate them. There were white deposits on its gastric mucosa, which seemed smoother and without the large folds observed in the group 3 animal. The texture of the kidneys was very firm and they were light in colour. The liver was hard and friable. The pericardium was sclerotic, and there were white intrapericardial deposits. There were multifocal calcifications of the subendothelial connective tissue of arteries in its heart (Fig 3), in the perirenal connective tissue, in the renal tubuli (Fig 4), and in the connective tissue of the lungs. The cortices of the femurs were thick.

DISCUSSION

These results provide the first objective assessment of the effects of supplementing the diet of captive tortoises with calcium. The metabolic and nutritional requirements of tortoises are closely related to their natural environment, and adequate housing and lighting conditions were provided so as not to influence the outcome. Juvenile animals were studied because they are growing more quickly and have higher calcium requirements than adults. The calcium and phosphorus content of most of the vegetables, fruits and greens which make up the conventional diet for captive herbivorous reptiles is poor compared with that of the grasses, mixed shrubbery, cacti and other plants they eat in the wild (Kabigumila 2001). Frye (1995) emphasised the large amounts of calcium needed for the proper development of the shell.

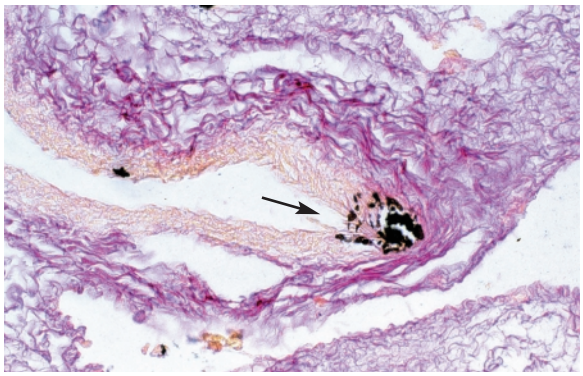

FIG 3: Subendothelial deposits of calcium salts (arrow) in the heart of the tortoise from group 4. Von Kossa. x **380**

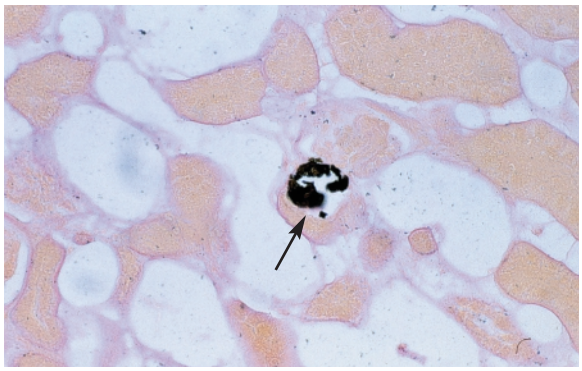

FIG 4: Calcium deposits (arrow) within a renal tubule of the tortoise from group 4. Von Kossa. x **380**

General clinical observation revealed no signs of dehydration during the study period. The tortoises with no or minimal calcium supplementation (groups 1 and 2) did not thrive. They exhibited typical signs of metabolic bone disease and were undersized for their age, and the shells of the tortoises of group 1 failed to calcify and remained soft. Scanning clearly showed that the tortoises of group 1 had a lower calcium content than those of groups 2 to 4 (Fig 2), probably as a result of the depletion of calcium reserves to maintain basic metabolic requirements. At the end of the study, the mean plasma calcium concentration of group 1 was significantly lower, and their levels of phosphorus and alkaline phosphatase were slightly higher than those of the other groups, probably in response to an increased secretion of parathyroid hormone, which acts to increase serum alkaline phosphatase and phosphorus (Mader 2000). Scott (1992) pointed out that nutritional secondary osteodystrophy is very easy to produce in young chelonians by feeding them deficient diets, and one of its manifestations is a soft shell. The carapace and plastron of the group 1 tortoises were as thin as paper and could be bent by hand.

The tortoises receiving the recommended calcium supplementation (group 2) had increased in weight by about 12 per cent, but the calcium content of their bodies did not increase significantly, and the shell of the tortoise examined postmortem was thin. However, the cortices of its femurs were more developed than those of the tortoise from group 1, and histology revealed focal areas of calcification of the connective tissue and in its renal tubuli and lungs. The evidence is conflicting, but the fact that its shell had not calcified fully suggests that it did not receive sufficient calcium supplementation. The results of group 2 were compromised by the fact that only three of the six tortoises survived.

The tortoises which were given three times the recommended calcium supplementation (group 3) were in a good state of health, and they had the highest growth rate. Scanning showed that there was a significant increase in their body calcium content. Postmortem, there were multifocal calcifications of the connective tissue in the lungs, focal areas of calcification in the heart, fatty degeneration of the liver, and the femurs had thick cortices, suggesting that calcium had been given in excess. The tortoises receiving nine times the recommended calcium supplementation (group 4) were also in a good state of health, but their growth rate was lower than that of groups 2 and 3, and scanning showed that there had been a significant increase in their body calcium. Focal calcification of the connective tissue was observed in the subendothelial connective tissue of vessels in the heart, perirenal connective tissue, renal tubuli and the connective tissue in the lungs; such metastatic calcinosis is the result of serum calcium and phosphorus being in a supersaturated state (Fowler 1978).

The postmortem findings are in accordance with Frye (1995), who emphasised that supplementing the diet of captive reptiles with vitamins and minerals can induce vitamin toxicities and imbalances between calcium, phosphorus and, to a smaller extent, magnesium and some trace minerals. The results of the present study indicate that plasma calcium concentration is of little diagnostic value, owing to the complex homeostatic mechanisms that control it. In contrast, scanning can detect calcium defects, define the condition of the skeleton, and determine the rate of increase of body calcium during the growth period.

Even though there was a difference in the size of the tortoises, scanning showed that all of them had approximately the same level of calcification and had a nearly dose-dependent rate of calcification during the study. The calcium supplementation of groups 3 and 4 was shown to have been excessive, as metastatic calcifications were observed, but calcium supplementation was shown to be necessary, as evidenced by inadequate calcification of the shells and skeletons of the tortoises in groups 1 and 2. The relative calcium deficiency observed could not be related to a rapid rate of growth due to high protein levels, because the diet was purely vegetable, and no shell distortions, such as pyramidal doming, were observed (Mas 2001).

The results of this study suggest that providing a mixture of chopped vegetables, powdered with the recommended calcium supplement of Diafarm, that is, 2·55 g calcium/kg fresh feed, may not meet the needs of captive leopard tortoises or other tortoises, if the basic diet is inherently deficient in calcium. However, the method of supplementation by means of calcium carbonate is the procedure normally followed when tortoises are kept in captivity, and this renders the results highly relevant to owners and veterinarians.

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