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Plasma concentrations of 25-hydroxycholecalciferol in 22 captive tortoises (*Testudo* species)

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The plasma concentration of 25-hydroxycholecalciferol was measured in 13 adult Hermann's tortoises (*Testudo hermanni*), seven adult spur-thighed tortoises (*Testudo graeca*) and two adult marginated tortoises (*Testudo marginata*) during 2004. They were healthy, of both sexes, and kept in captivity under natural unfiltered sunlight in southern England with no dietary sources of cholecalciferol. Blood samples were taken in March, June and August, and the concentration of 25-hydroxycholecalciferol did not vary significantly with the seasons. However, the concentrations in the female tortoises were always significantly lower than in the males.

DISORDERS of calcium metabolism are commonly reported as being a cause of morbidity and mortality in captive reptiles. They are usually the result of dietary calcium deficiency, imbalances in the dietary calcium:phosphorus ratio or hypo- or hypervitaminosis D. A lack of vitamin D (cholecalciferol) can be due either to a dietary deficiency or to a failure to provide adequate UVB radiation, either naturally or artificially (Boyer 1996, Calvert 2004). Cases of vitamin D toxicity have also been described (Burgmann and others 1993, Calvert 2004).

It is important that vitamin D_3 precursors and calcium are available in the diet. It has been shown in rats that low levels of dietary calcium can reduce the plasma concentration of 25-hydroxycholecalciferol (25-HCC) by increasing its rate of inactivation by the liver (Clements and others 1987). The levels of dietary calcium required remain largely unknown but values approximating 1 per cent of dry matter intake have been reported for a variety of reptiles (Calvert 2004). These requirements will vary with the animals' life stage and reproductive activity; growth and reproduction will increase the requirement whereas animals with good bone stores of calcium will have lower requirements (Calvert 2004).

Vitamin D deficiency may develop in reptiles kept at higher latitudes or under inadequate artificial sources of UVB radiation. Reptiles given diets high in vitamin D_3 have been reported to suffer from calcium disorders, and these may be due to species-specific differences in their ability to absorb and utilise oral vitamin D_3 (Calvert 2004). The failure of oral vitamin D_3 to meet animals' requirements has been reported by Ullrey and Bernard (1999), who describe how a dietary bolus of vitamin D_3 failed to maintain the blood 25-HCC levels of green iguanas (*Iguana iguana*) for five weeks; exposing them to an artificial source of UVB was far more effective. Allen and others (1995) investigated vitamin D_3 metabolism in day geckos (*Phelsuma madagascariensis*) and leopard geckos (*Eublepharis macularius*) and found that when they were provided with oral vitamin D_3 but deprived of UVB radiation leopard geckos had normal bone growth but day geckos did not. Dietary supplements contain calcium in different forms and variable quantities of phosphorus and cholecalciferol, and it is very easy for captive reptiles to be either under or over supplemented.

The synthesise of metabolically active vitamin D_3 requires exposure to UVB radiation (315 nm to 290 nm), by exposure either to sunlight or to lamps producing UVB. Provitamin D_3 (7-dehydrocholesterol) is converted by UVB radiation to previtamin D_3, which undergoes thermal transformation to cholecalciferol. This has been demonstrated in green iguanas by Holick and others (1995). As a result, environmental temperature plays an important role in vitamin D formation. Furthermore, not all of the available UVB wavelengths convert provitamin D to previtamin D with the same efficiency, peak conversion has been reported (in a variety of species) at between 303 nm and 295 nm (Ullrey and Bernard 1999). Exposure to sunlight (and hence other wavelengths) leads to a quasiphotostationary state between provitamin D, previtamin D, lumisterol and tachysterol (Holick and others 1981).

There have been no reports of vitamin D toxicity as a result of exposure to sunlight, possibly owing to the photoisomerisation of previtamin D_3 to lumisterol and tachysterol by UVB. This process is reversible and these compounds can act as a store for cholecalciferol, moreover excess UVB can degrade cholecalciferol to inert compounds (Ferguson and others 2003).

The cholecalciferol produced by UVB enters the circulation where it is transported to the liver and converted to 25-HCC. It has been reported that this step is rapid and largely unregulated resulting in variable blood levels (Johnson and Ivey 2002, Ferguson and others 2003). However, there is evidence in human beings and rats that the levels of 25-HCC are regulated by product feedback (Bell and others 1984, Milne and Baran 1985). 25-HCC is transported to the kidneys where it is transformed to 1,25-dihydroxycholecalciferol (DHCC). This step is reported to be much slower and subject to product feedback control (Johnson and Ivey 2002). The blood levels of 25-HCC can vary and depend on the rate of production from dietary precursors, exposure to UVB and the demand for DHCC. There is tight regulation of DHCC by product feedback and its levels are kept within a narrow range (Holick 1999). The blood levels of 25-HCC should help to determine how much exposure there has been to UVB radiation and the level of vitamin D precursors in the diet. In human beings, the half-life of 25-HCC is 15 to 21 days (Holick 1999), and its blood level represents the balance between its rate of photobiogenesis and the level of vitamin D in the diet, and its rate of metabolism (Ullrey and Bernard 1999, Gyimesi and Burns 2002). Studies in panther chameleons (*Furcifer pardalis*) have shown that basking in UVB radiation significantly increases the circulating levels of 25-HCC (Ferguson and others 2003).

In all vertebrates, 25-HCC is stored in the liver, but little is known about the process in reptiles. However, there is evidence that their liver has a poor storage capability (Ullrey and Bernard 1999), and there is some evidence of short-term storage in Komodo dragons (*Varanus komodoensis*) (Gyimesi and Burns 2002). The plasma levels of 25-HCC vary between species of reptiles (Bernard 1995, Laing and others 1998, Gillespie and others 2000, Dennis and others 2001, Gyimesi and Burns 2002, Nevarez and others 2002, Ramer and oth-

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TABLE 1: Mean daily exposure of the tortoises to UVB radiation during the three weeks before the blood samples were taken, and the average daily mean, maximum and minimum temperatures during each month

Month	Daily UVB exposure* $\times 10^6$ KJ/m^2	Temperature (°C)† Maximum	Minimum	Mean
March	1·128	10·3	2·9	6·6
June	2·743	20·9	10·9	15·9
August	1·777	22·6	13·5	18·0

* National Radiological Protection Board data
† Met Office data

ers 2002, 2005, Aucone and others 2003, Mitchell and others 2005).

Although *Testudo* species inhabit temperate regions, the latitudes from which they originated are closer to the equator than the UK and latitude has a marked effect on potential exposure to UV radiation from the sun. The temperature ranges are also significantly different. In the winter, UK temperatures may not be very different from those in their natural range, but in summer the temperatures and potential exposure to UVB radiation are much lower. Exposure to UVB also varies over the course of the year and is highest during the summer months.

It has been reported that gravid reptiles bask for longer periods (Hernandez-Divers 2001, Ferguson and others 2003). This not only raises their temperature (and makes metabolic processes more efficient) but also increases the rate of production of previtamin D_3 and hence 25-HCC. It has been suggested that increased basking satisfies a need for increased calcium and vitamin D_3 during folliculogenesis, and that vitamin D_3 production is an important factor in stimulating diurnal reptiles to bask (Ferguson and others 2002, 2003). Higher plasma concentrations of 25-HCC have been recorded in gravid female green iguanas than in non-gravid females (Nevarez and others 2002).

The scientific literature on the calcium metabolism of chelonians is sparse and much remains unknown or is derived speculatively from studies of other species. The aim of this study was to determine the plasma concentration of 25-HCC in tortoises under defined environmental conditions and whether there were variations with season or differences between males and females.

MATERIALS AND METHODS

Thirteen Hermann's tortoises (*Testudo hermanni*), seven spur-thighed tortoises (*Testudo graeca*) and two marginated tortoises (*Testudo marginata*) undergoing routine health examinations were studied. They received no artificial light but were exposed to unfiltered sunlight in Chippenham, southern England (51° 30'N) during the summer months and hibernated during the winter. Their diet consisted of kale, cauliflower, spring greens, cabbage, lettuce, endive, watercress, clover, radish leaves, tomatoes, cucumber, melon, strawberries, dandelion leaves and sow thistle leaves, supplemented with calcium carbonate. The diet had a calcium: phosphorus ratio of approximately 7·4:1 and the calcium constituted 2·4 per cent of the dry matter; it contained 20·7 per cent protein, 5·2 per cent fat and 45·6 per cent crude fibre, on the basis of a computer-based feed analysis programme (Zootrition, version 2; Wildlife Conservation Society). There were no dietary sources of cholecalciferol. Supplementary heating was provided immediately after the tortoises emerged from hibernation until the weather was sufficiently warm for them to remain permanently outside. All the animals were of reproductive age and had hibernated successfully. They were clinically examined before the start of the study, and any considered ill or unhealthy were rejected and treated appropriately. Jugular blood samples were taken for haematology and blood biochemistry after their emergence from hibernation to establish that they were healthy and further blood samples were taken during the year. A final prehibernation sample was taken to assess their status and suitability for hibernation. A total of 22 tortoises satisfied these selection criteria, but blood samples were not obtained from all of them on each occasion.

The blood samples (0·5 to 1 ml) were collected into heparin-containing tubes and stored as heparinised whole blood at 5°C until analysed biochemically and haematologically within 48 hours. Samples were taken in March (immediately after hibernation and before they had been exposed to natural light), June and August (the prehibernation sample). Plasma was separated by centrifugation and stored at 5°C until batch analysed for 25-HCC using an enzyme immunoassay (Lind and others 1997). Each sample was diluted with biotin-labelled 25-HCC and added to microtitre wells coated with sheep antibody to 25-HCC. The sample was then incubated and washed. Peroxidase-tagged avidin was added, which binds selectively to the complexed biotin.

Statistical analyses

The mean (se) of each data set was calculated and the data were tested for normality using the Shapiro-Wilks test. Non-normally distributed data were analysed by non-parametric tests; analyses between paired samples were made using the Wilcoxon signed-rank test, and analyses between independent samples were made using the Kruskal-Wallis one-way analysis of variance. Comparisons were made between the results for the sexes in each month, and between the months. The analyses were made by using the program. Excel Analyse It (Microsoft), with significance being accepted when $P<0·05$.

TABLE 2: Mean (se) plasma concentrations of 25-hydroxycholecalciferol (nmol/l) in samples taken from male and female tortoises at different seasons

Sample	Sex (n)	Mean (se)	95% confidence intervals	P for differences between males and females*
All dates	Both (56)	28·41 (2·00)	24·41-32·42	
	Males (23)	38·61 (3·28)	31·80-45·42	<0·0001
	Females (33)	21·30 (1·62)	18·00-24·61	
March	Both (18)	26·61 (2·90)	20·49-32·74	
	Males (6)	33·50 (4·26)	22·54-44·46	0·0545
	Females (12)	23·17 (3·49)	15·49-30·85	
June	Both (17)	29·82 (5·11)	19·00-40·65	
	Males (8)	42·38 (8·90)	21·33-63·42	0·0080
	Females (9)	18·67 (1·96)	14·15-23·18	
August	Both (21)	28·81 (2·45)	23·70-33·93	
	Males (9)	389·67 (1·67)	34·82-42·51	0·0002
	Females (12)	21·42 (2·46)	16·01-26·83	

* There were no significant differences between the samples taken in different months

RESULTS

None of the tortoises became ill or died during the study, and they all tolerated venepuncture well.

No significance signs of disease were observed and the results of the blood biochemical analyses and haematological profiles were within reference ranges reported previously (data not shown). The mean daily solar UVB radiation received by the tortoises during the three weeks before the blood samples were taken and the average mean, maximum and minimum daily temperatures throughout each month are shown in Table 1.

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The level of solar UVB was highest before the samples taken in June but the environmental temperatures were highest in August.

Table 2 shows the mean (se) concentrations of 25-HCC in the samples analysed in March, June and August, and their 95 per cent confidence intervals. Different numbers of tortoises were sampled in each month.

The concentrations did not differ significantly between the months, but the males had significantly higher values over the whole year, and in June and August samples specifically.

DISCUSSION

Reference ranges for 25-HCC levels have not been defined in reptiles. In a study of desert tortoises (*Gopherus agassizii*) housed outdoors in Nevada, USA, values ranging between 12·5 nmol/l and 41·3 nmol/l were recorded. Juvenile sulcata tortoises (*Geochelone sulcata*) and desert tortoises housed indoors and fed on diets containing vitamin D_3 had values less than 12·5 nmol/l (Ullrey and Bernard 1999). Measurements in adult green iguanas showed no statistically significant differences between the concentrations in males (mean 188·9 nmol/l, range 51·1 to 326·7 nmol/l), females (mean 233·8 nmol/l, range 74·8 to 392·8 nmol/l) and gravid females (mean 264·3 nmol/l, range 121·9 to 406·7 nmol/l). It was suggested that the slightly higher concentrations in the gravid females may have been due to an increased demand to mobilise calcium during folliculogenesis (Nevarez and others 2002). Values in wild chuckwallas (*Sauromalus obesus*) (mean 211 nmol/l, range 136 to 286 nmol/l), Mali uromastyx (*Uromastyx maliensis*) housed outside in El Salvador (range 62·4 to 406·8 nmol/l) and wild Ricord's iguana (*Cyclura ricordii*) (mean 554 nmol/l, range 250 to 1118 nmol/l) and wild rhinoceros iguanas (*Cyclura cornuta*) (mean 332 nmol/l, range 260 to 369 nmol/l) have been reported (Ramer and others 2002, 2005, Aucone and others 2003, Mitchell and others 2005). Serial analyses of 25-HCC in Komodo dragons showed that they had higher blood concentrations after exposure to sunlight or active UVB lamps (Gillespie and others 2000, Gyimesi and Burns 2002), and similar results have been observed in chuckwallas, however, traditional fluorescent tubes resulted in lower values than in wild-caught individuals (Aucone and others 2003).

The concentrations of 25-HCC measured in this study are comparable to those previously reported in tortoises housed indoors (Ullrey and Bernard 1999). The concentrations did not vary with season, despite the variations in UVB radiation and temperature, suggesting that the seasonal variations in solar radiation and environmental temperature in the UK probably have little effect on the photobiogenesis of previtamin D.

The use of artificial UVB sources should be considered when tortoises are kept at higher latitudes, if it is found that the concentrations of 25-HCC in wild tortoises are similar to those of lizards (Gillespie and others 2000, Dennis and others 2001, Gyimesi and Burns 2002, Ramer and others 2002, 2005, Aucone and others 2003, Mitchell and others 2005). Dietary supplementation with vitamin D is another possible method for raising blood levels of 25-HCC, but many basking reptile species require UVB lighting to maintain their blood 25-HCC levels and dietary sources of vitamin D alone may be inadequate (Allen and others 1999, Ullrey and Bernard 1999). It is uncertain whether chelonians can utilise oral vitamin D, but Ullrey and Bernard (1999) recorded low blood levels despite oral supplementation.

It has been shown that environmental temperature affects the rate of vitamin D production in green iguanas (Holick and others 1995), but it is not known to what extent temperature influences vitamin D production in chelonians. It is assumed that higher temperatures are required to stimulate adequate production, and the current recommendation for captive basking species of chelonians is to provide a basking site at the higher end of the activity temperature range in combination with the UVB source (Adkins and others 2003).

The relative importance of environmental temperature, dietary vitamin D and artificial UVB sources on the plasma 25-HCC levels in captive chelonians needs to be evaluated further to identify whether there are any shortcomings in current husbandry practices. The measurements of other parameters, such as parathyroid hormone may provide useful information when assessing their calcium metabolism.

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