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CHARACTERIZING THE 25-HYDROXYVITAMIN D STATUS OF TWO POPULATIONS OF FREE-RANGING EASTERN BOX TURTLES (*TERRAPENE CAROLINA CAROLINA*)

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Abstract: Ultraviolet B radiation is recommended for captive reptiles to stimulate production of adequate levels of vitamin D; however, little is known regarding the vitamin D status in many free-ranging populations. Current reference ranges for vitamin D in eastern box turtles have not yet been established. Sixty free-ranging eastern box turtles (*Terrapene carolina carolina*) from two well-studied populations in Illinois ($n = 24$) and Tennessee ($n = 36$) were assayed for plasma vitamin D concentration in 2014. There were no significant differences in concentrations between individuals in Illinois (mean: 117.5 nM/L) and Tennessee (mean: 98.7 nM/L) ($P = 0.129$) populations. Similarly, there were no differences in concentrations based on age class ($P = 0.533$) or sex ($P = 0.532$). There was a significant correlation between UV at the time of capture and vitamin D concentrations ($R = 0.301$, $P = 0.030$). Vitamin D was not correlated with total calcium ($R = 0.018$, $P = 0.89$) or Ca : P ratio ($R = 0.025$, $P = 0.85$). Diseases in captive individuals, including secondary nutritional hyperparathyroidism, may commonly be associated with vitamin D deficiencies, and clinical intervention relies on reference data. Vitamin D supplementation may be recommended if animals are deemed to be deficient. Data obtained can be used to improve the care of captive and free-ranging turtles by providing reference ranges, as well as better characterize the health of wild populations.

Key words: 25-hydroxyvitamin D, chelonian, eastern box turtle, free-ranging, *Terrapene carolina carolina*.

INTRODUCTION

Vitamin D is commonly known as a fat-soluble vitamin, but also functions as a circulating hormone. It is a major component of homeostatic mechanisms, including bone development, growth, neuromuscular function, reproduction, cardiovascular health, and immune function across species. In humans, inadequate levels of vitamin D have been shown to increase the risk of developing a number of different diseases, including diabetes, muscular dystrophy, hypertension, and inflammatory bowel disease.[6] In reptiles, inadequate levels of vitamin D will affect calcium absorption and ultimately lead to development of secondary nutritional hyperparathyroidism. The role of vitamin D in reptile immune function is unknown; however, it is well-known that T and B cells play an important role in the adaptive immune response.[19] In other species, vitamin D receptors are present in activated T and B cells.[10] Thus, inadequate levels of vitamin D may affect immune response, thus increasing the risk or altering severity of common pathogens threatening animal and/or population health.

The eastern box turtle (*Terrapene carolina carolina*) is a common captive reptile and one of the few terrestrial chelonians in the eastern United States. It is listed as vulnerable by the International Union for Conservation of Nature red list (IUCN 2013) and as an Appendix II species of the Convention on the International Trade of Endangered Species (CITES). Several factors are leading to its conservation threat, including infectious diseases such as ranavirus, herpesvirus, and Mycoplasma,[3,4,6] and any attempts to identify parameters that may have a negative effect on their overall health are important.

Most omnivorous reptiles obtain vitamin D directly from their diet or secondary to exposure to ultraviolet B (UVB) radiation. Synthesis of vitamin D is the result of the photosynthetic conversion of 7-dehydrocholesterol (provitamin D_3) to previtamin D_3 in the skin after exposure to UVB. Previtamin D_3 undergoes an isomerization

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into vitamin D_3, which has been shown to be enhanced at higher temperatures in poikilothermic animals such as iguanas and amphibians, as well as in humans.[15] Measuring plasma concentrations of 25-OHD_3 has been recommended as the preferred method of characterizing the vitamin D status in the body because it is more stable than either vitamin D or calcitriol, has a longer half-life than other metabolites,[9] and represents the overall average concentration of dietary and sunlight-induced vitamin D in the body.[12] Only radiation in the UVB wavelengths of 280–320 nm will allow for this precursor conversion in the skin. The narrow range of sufficient wavelengths may imply that habitat and location play an important role in vitamin D synthesis. The UV index of natural sunlight is highly variable based on latitude, time of day, and time of year. Living at higher latitudes has been found to increase the risk of hypertension and cardiovascular disease in humans, which has been correlated to lower circulating levels of vitamin D.[11,20]

The objective of this study was to evaluate vitamin D status in two well-studied populations of eastern box turtles (Illinois and Tennessee) and characterize differences based on location, sex, age class, clinical pathology, and UV index taken at the time of capture.

Our hypothesis is that the Tennessee population will have significantly higher 25-hydroxyvitamin D levels than the Illinois population due to the lower latitude of the former.

MATERIALS AND METHODS

Individual box turtles were collected from two free-ranging populations in Illinois and Tennessee as part of an ongoing health assessment during July 2014 using human and canine turtle searches. While multiple sites were used in each state, sites were less than 60 miles away from laboratory locations in Urbana, Illinois (40.1097°N, 88.2042°W) or Oak Ridge, Tennessee (35.9728°N, 83.9422°W), respectively. This project was approved and performed in accordance with the regulations established by the Institutional Animal Care and Use Committee at the University of Illinois (protocol 13061).

UVB radiation was measured in microwatts per centimeter squared ($\mu W/cm^2$) by placing a digital UVB meter (Solartech, Inc., Harrison Township, Michigan 48045, USA) and an irradiance meter (Zoo Med Laboratories Inc., San Luis Obispo, California 93401, USA) on the ground at the exact level and location the turtle was identified.

Once turtles were collected, a physical examination was performed; age, sex class, and mass was recorded; and a blood sample was collected. Blood samples were collected from the subcarapacial sinus using a 22-ga needle attached to a 3-ml syringe. The blood sample volume was less than 1% of the total body weight of the animal. The samples were placed in lithium heparin vacutainers (Becton, Dickinson, and Company, Franklin Lakes, New Jersey 07417, USA). The vacutainer tubes were centrifuged for 10 min within 2 hr of collection. Plasma was harvested and subsequently placed into cryovials and stored at −20°C.

Sample size was determined using an α of 0.05, and a β of 0.2 with an expected difference in 25-OHD_3 of 15 nM/L, with a standard deviation of 20 nM/L based on previous studies comparing populations with and without UVB supplementation.[1,2,5,23] Using these parameters, this gave a necessary sample size of 58 turtles to detect a difference in vitamin D concentration between the populations if one exists (nondirectional two-tail). Plasma samples were previously collected and stored at −80°C. Plasma samples were submitted to a veterinary diagnostic laboratory (Diagnostic Center for Population and Animal Health, East Lansing, Michigan 48910, USA) for measurement of plasma 25-hydroxyvitamin D (25-OHD_3, radioimmunoassay).

Plasma electrolytes (Ca, P, and Ca : P ratio) were concurrently analyzed as part of the ongoing health assessment and results were available to determine if plasma chemistry values were correlated to vitamin D. These plasma samples were collected from the same aliquots as those used in this study. Briefly, all biochemical analytes were measured in the University of Illinois Clinical Pathology Lab using a Beckman Coulter AU680.

Statistical analysis

Data was evaluated for normality using q-q plots, skewness, kurtosis, and the Shapiro-Wilk test. Mean, SD, and minimum–maximum (min–max) values were reported for data that had a normal distribution, whereas the median, 10th to 90th percentiles (%), and minimum–maximum (min–max) values were reported for data that did not have a normal distribution. A *t*-test or Mann-Whitney *U*-test was performed to determine differences in vitamin D levels between locations, age class, and sex for normally and nonnormally distributed data, respectively.

Bivariate correlation was used to assess association between vitamin D and UV and biochemical analytes. Data was analyzed by commercial software (SPSS 22.0, SPSS Inc., Chicago, Illinois 60606, USA). A P value of less than 0.05 was considered significant.

RESULTS

This study measured vitamin D status in 60 individuals from two populations of eastern box turtles in Illinois ($n = 24$) and Tennessee ($n = 36$). 25-OHD$_3$ concentrations in plasma were not significantly different between Illinois (mean: 118 ± 51 nM/L, min–max: 25–199) and Tennessee (mean: 99 ± 43 nM/L, min–max: 25–231) populations (Fig. 1A).

The number of individuals represented by each variable and by population is represented in Table 1. There were no differences in 25-OHD$_3$ status between age class or sex. Vitamin D status between population, sex, and age class is represented in Table 2. There was a significant correlation between UV at the time of capture and 25-OHD$_3$ concentrations ($R = 0.301$, $P = 0.030$; Fig. 1B). There was a significant difference between UV level and location ($P < 0.0005$). UV levels in Illinois (5.0 ± 8.4 μW/cm^2, min–max: 1–30) were higher than Tennessee (0.0 ± 0.04 μW/cm^2, min–max: 0.0–0.1). 25-OHD$_3$ was not correlated with total calcium ($R = 0.018$, $P = 0.89$) or Ca : P ratio ($R = 0.025$, $P = 0.85$). Calcium (median: 11.4 mg/dl, min–max: 8.4–26.3, 10–90th percentiles: 9.5–19.5), phosphorus (median: 4 mg/dl, min–max: 1.8–7, 10-90th percentiles: 2.9–4.9), and calcium : phosphorus (Ca : P) ratios (median: 3.2, min–max: 2–5.6, 10–90th percentiles: 2.5–4.3) were all within normal limits.

DISCUSSION

This study set out to characterize vitamin D status in two populations of eastern box turtles, varying by latitude. Different determinants limit the cutaneous production of previtamin D$_3$ in humans, including photobiochemical regulation, pigmentation, and latitude, in order of importance.[14] For this reason, it was hypothesized that the Tennessee population located at lower latitude, closer to the equator, would have higher vitamin D levels. However, there was no difference in vitamin D between populations, suggesting either that the difference in latitude was not large enough to cause a significant difference, or that latitude does not have an effect on vitamin D production in eastern box turtles. Consistent with our findings, a meta-analysis performed evaluating vitamin D levels in relation to latitude in humans did not find a difference associated with latitude.[8] However, other publications have shown that latitude does alter vitamin D in humans.[13,18] While latitude may play a role in vitamin D production, the metabolism of a reptile is dependent on sunlight and ambient temperature. It should be considered that it is difficult to make direct comparisons between humans and reptiles, due to drastic differences in metabolism, behavior, and environment.

Within the two populations of turtles, there was a wide range of 25-OHD$_3$ levels. The overall range between populations was 25–231 nM/L. Findings in this study are higher than the ranges documented in wild green sea turtles (min–max: 16.1–72.1) nM/L.[22] Since eastern box turtles are terrestrial chelonians and basking behavior differs from aquatic green sea turtles, this may account for the difference in mean and maximum 25-OHD$_3$. In humans, a serum 25-OHD$_3$ concentration of <50 nM/L is considered deficient.[11] However, it should be noted that this minimum value is a general recommendation by most experts, and a general consensus on optimum levels of 25-OHD$_3$ in humans does not exist. While the mean plus one standard deviation falls above this cutoff, by these recommendations the turtles that fell on the lower end of the range within the population may be deficient. No physical examination or electrolyte abnormalities were noted in these individuals. Future studies are needed to establish direct correlation with hypovitaminosis D and clinical disease in free-ranging populations.

There was an expected positive correlation between 25-OHD$_3$ level and UVB level at the time of capture. Previous studies have shown that red-eared sliders (*Trachemys scripta elegans*) possess the capability of increasing 25-OHD$_3$ levels after exposure to artificial UVB radiation.[1] Therefore, a higher UVB level should increase circulating vitamin D. Although not yet studied in wild populations of eastern box turtles, many omnivorous reptile species have been shown to have the ability to obtain 25-OHD$_3$ from both their diet and exposure to UVB radiation. In fact, there has been debate as to whether vitamin D$_3$ produced in the skin is equivalent to that found in dietary sources. Both seem to have the same biologic activity; however, the half-life of vitamin D$_3$ produced in the skin is much longer because it is 100% bound to vitamin D binding protein. Conversely, only about 60% of dietary vitamin D$_3$ is bound in circulation.[7] This may suggest that circulating 25-OHD$_3$ may be more representative

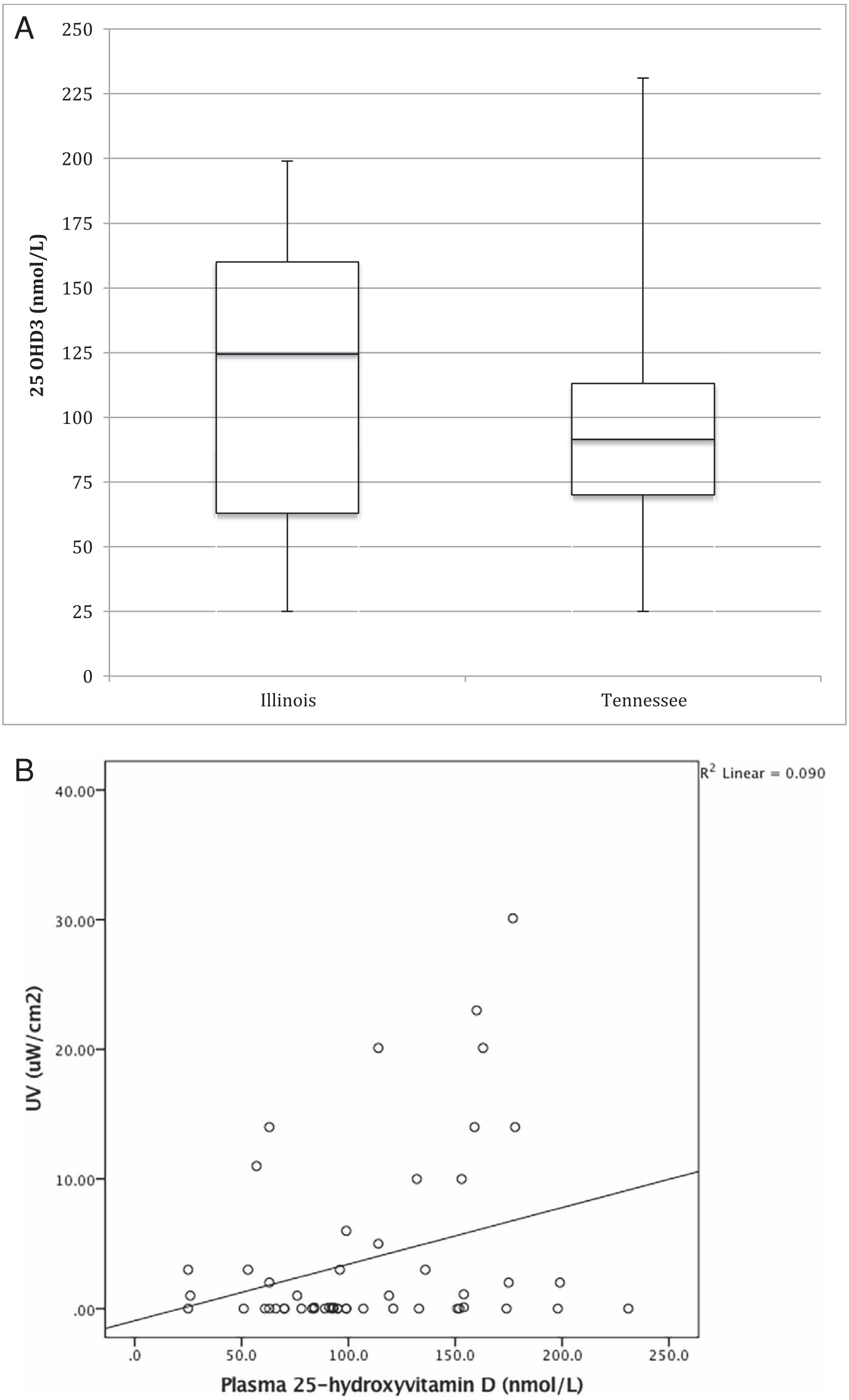

Figure 1. **A.** Distribution of 25-OHD$_3$ levels (nM/L) by location. Although Tennessee turtles appear slightly lower, the difference was not statistically significant. **B.** Correlation between UV (µwatts/cm^2) at the time of capture and 25-OHD$_3$ concentrations (nM/L).

Table 1. Number of individuals represented by each variable and by population.

Population	Females	Males	Unknown sex	Adult	Juvenile
Illinois	9	14	1	22	2
Tennessee	20	14	0	35	0

Table 2. Plasma 25-hydroxyvitamin D concentrations in Eastern box turtles by location, sex (unknown sex not listed), and age class.

Variable	Plasma 25-hydroxyvitamin D concentration (nM/L)	
	Mean ± SD	Minimum–maximum
Populations		
Illinois (*n* = 24)	118 ± 51	25–199
Tennessee (*n* = 35)	99 ± 43	25–231
Sex		
Female (*n* = 29)	103 ± 43	25–178
Male (*n* = 28)	110 ± 44	26–199
Unknown (*n* = 1)		
Age class		
Adult (*n* = 57)	106 ± 47	25–231
Juvenile (*n* = 2)	128 ± 45	96–159

of recent UVB exposure and basking behavior. Since diet was not analyzed, it is unknown how much exposure to UVB radiation contributed to vitamin D levels versus diet. UV levels were significantly higher in Illinois than Tennessee. However, there was no significant difference in vitamin D levels between populations, despite a significant difference in UV radiation recorded at the time of sampling. In Illinois, the UV levels may have been higher because the habitats are more variable and include open fields, whereas the Tennessee field site consists only of forest. Turtles in environments with higher UVB may self-regulate exposure or diet to maintain the vitamin D concentrations within an optimal range.

UVB radiation and subsequent vitamin D production has been found to significantly affect behavior and physiologic processes in many reptile species. A significant increase in 25-OHD_3 concentrations secondary to UVB exposure has been observed in both omnivorous and herbivorous chelonians.[1,21] Vitamin D may be the driving force of basking behavior in some species of chelonians.[17] It is not known if this is true for eastern box turtles. Eastern box turtles naturally burrow and may not spend a large amount of time basking. Although UV levels were recorded at the time of capture where the animal was found (e.g.. in water, shade, open field, etc.), these levels may not necessarily be directly representative of the recent basking status of the turtle. Future studies may include investigating the half-life of circulating vitamin D in box turtles as well as determining if there is a difference in vitamin D depending on season.

Vitamin D also contributes to mobilizing calcium from the GI tract, so it might be postulated that increased vitamin D would increase calcium levels. However, calcium levels and Ca : P ratios were not correlated to 25-OHD_3 in this study. Since calcium levels obtained were considered normal, this suggests that the overall calcium status of the animals in this study was stable with no increased physiologic need to mobilize calcium. Circulating vitamin D was likely contributing to normal calcium homeostasis.

Several studies have evaluated the overall health of eastern box turtles, but to our knowledge vitamin D has never been investigated in box turtles either in captivity or in a free-ranging population. The role of vitamin D in the health of chelonians, especially in regards to infectious disease, is largely unknown. Studies in cats have demonstrated that deficiencies of vitamin D levels are associated with active mycobacteria infections,[16] suggesting vitamin D may play an important role in preventing disease or modulating immune function when fighting an infection in some animals. Future studies may include assessing vitamin D status in individuals with known pathogen prevalence.

In conclusion, 25-OHD_3 levels were similar between eastern box turtle populations in this study and correlated to UVB level at time of capture. 25-OHD_3 has not previously been studied in free-ranging box turtle populations. Since all animals were apparently healthy based on physical examination and complete blood counts and plasma biochemistry values, these values also provide guidelines for 25-OHD_3 levels in free-ranging populations of box turtles. Data obtained can be used to improve the care of captive and free-ranging turtles.

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