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Article:

SEASONAL VARIATION OF SERUM 25-HYDROXY-VITAMIN D IN TWO CAPTIVE EASTERN BLACK RHINOCEROS (*DICEROS BICORNIS MICHAELI*) HOUSED IN A NORTH AMERICAN ZOO

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Abstract: Black rhinoceros (*Diceros bicornis* spp.) are critically endangered species, with less than 65 individual animals housed in captivity within Association of Zoos and Aquariums-accredited zoos within the United States, and an estimated 5,500 individual animals of all subspecies surviving in the wild. Previously published reference values for circulating vitamin D_3 (25OHD$_3$; 55.7 ± 34.2 ng/ml) were based upon samples from free-ranging black rhinoceros in Africa. Recent research in human medicine has highlighted the importance of subclinical vitamin D deficiency, with links to increased risks for developing various health conditions. Serum samples collected opportunistically from two captive Eastern black rhinoceros (*Diceros bicornis michaeli*) housed with seasonal access outdoors in a North American zoo were tested for 25-hydroxy-vitamin D (25OHD) levels over a 3-yr period. A commercially prepared pelleted diet containing vitamin D_3 was fed to both rhinos. This study correlates environmental ultraviolet (UV) index, dietary supplementation, and seasonal serum 25OHD levels to compare with known 25OHD$_3$ levels in free-ranging African black rhinoceros. Results in these two individuals suggest that *D. bicornis* spp. are dependent upon sunlight or UVB for measurable circulating 25OHD, and that current vitamin D_3 supplementation levels may have little effect for *Diceros* spp. in human care housed in northern latitudes.

Key words: Black rhinoceros, *Diceros bicornis michaeli*, 25-hydroxy-vitamin D, 25OHD, UVB.

INTRODUCTION

Vitamin D is a fat-soluble vitamin with important functions in metabolic pathways and contributions to immune system enhancement for both humans and animals. In humans, suboptimal levels of vitamin D have been linked to increased risks for immune system disorders, septicemia, cancers, lowered fertility rates, obesity, autoimmune disease, lessened ability to produce adequate calcium levels necessary for fetal growth, and cardiovascular diseases.[8] Although the exact etiology for many diseases affecting black rhinoceros (*Diceros bicornis* spp.) is yet unknown, black rhinoceros in human care suffer from many disease syndromes that have not been documented in free-ranging populations.[24] Understanding the influence of environment and nutrition for black rhinoceros in human care is critical to long-term health and survival.

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Vitamin D is found in two main forms, ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3). Ergocalciferol is naturally found in plants, whereas cholecalciferol is produced endogenously by an animal's skin. For an animal to produce vitamin D_3, skin must receive unobstructed contact with the ultraviolet B (UVB) radiation found in natural sunlight. UVB, in wavelengths between 280 and 315 nm, will not pass efficiently through windows or other barriers. The level of sunlight and UVB radiation that an animal receives is also affected by distance from the equator, season of the year, and time of day.[22] Black rhinoceros are naturally found in central to southern Africa between the latitudes of 15°N and 34°S.[5]

Vitamin D_3 is produced in the skin by UV light (290–315 nm) photolytic-induced cleavage of 7-dehydrocholesterol (7-DHC), producing previtamin D_3, which forms vitamin D_3 after thermal isomerization. Circulating vitamin D_3—whether ingested as vitamin D_3 in the diet or synthesized by the animal—can then be converted to 25-hydroxy-vitamin D_3 (25OHD$_3$) in the liver. This 25OHD$_3$ then circulates and can be further modified to 1,25-dihydroxy-vitamin-D_3 in the kidneys, under parathyroid hormone stimulation, to help maintain calcium homeostasis.[29] Vitamins D_2 and D_3, as well as all of their metabolites, have different efficacies and half-lives, which lead to slightly different functions. Vitamin D_3 is at least

Table 1. Labeled vitamin D_3 content, amount, and dates of commercial pellets fed to Blank Park Zoo rhinoceros from January 2013 to May 2016.

Rhino	Date	Variety of pellet fed	Labeled vitamin D_3 content (IU/kg feed)	Estimated vitamin D_3 consumed in feed (total IU)[a]
A	01 Jan 2013 to 13 Mar 2014	Mazuri® wild herbivore	1,320	6,006[b]
	14 Mar 2014 to 03 Apr 2014	Mazuri wild herbivore	1,320	6,006
		Mazuri browser rhino	1,250	
	04 Apr 2014 to 23 May 2016	Mazuri browser rhino	1,250	5,113–5,688
B	01 Jan 2013 to 04 Apr 2013	HMS® ADF-16	1,200	1,632[b]
	05 Apr 2013 to 13 Mar 2014	HMS ADF-16	1,200	3,925–4,519
		Mazuri wild herbivore	1,320	
	14 Mar 2014 to 03 Apr 2014	HMS ADF-16	1,200	4,523–4,538
		Mazuri wild herbivore	1,320	
		Mazuri browser rhino	1,250	
	04 Apr 2014 to 23 May 2016	Mazuri browser rhino	1,250	5,113–6,250

[a] International units of vitamin D_3 estimated by number of kilograms of feed offered during time periods shown, and transition between different diets within time periods.
[b] Total estimated amount of vitamin D_3 in feed does not include supplemented oral tablets offered in March 2013.

three times as effective as vitamin D_2 in increasing serum 25-hydroxy-vitamin D (25OHD) levels. Circulating 25OHD is regarded as the best practical indicator of bodily vitamin D levels, and includes both the D_2 and D_3 metabolites of 25OHD.[1]

Only one published study has identified serum concentrations of $25OHD_3$ levels of 55.7 ± 34.2 ng/ml in a sample ($n = 28$) of free-ranging black rhinoceros obtained during translocation operations in Zimbabwe.[9] In that publication, $25OHD_3$ was measured by vitamin D-binding protein assay. The season of the year of sample collection was not specified. In that same study, serum levels for $25OHD_3$ were documented for only two captive animals (0.096 ± 0 ng/ml), distinctively lower than the levels of the free-ranging animals. Again, season of the year and method of testing were not presented for those captive rhinoceros levels.[9] Because of the apparent differences between published free-ranging and captive *Diceros* spp. vitamin D levels, this project sought to correlate exposure to natural sunlight with circulating 25OHD levels using current assay methods that may be more precise in two captive black rhinoceros housed in a midwestern North American zoo.

MATERIALS AND METHODS

Animals

Two Eastern black rhinoceros (*Diceros bicornis michaeli*) arrived at the Blank Park Zoo (BPZ) in Des Moines, IA (41°N) during the first week of December 2012. The female rhinoceros was born in Miami, FL (25°45′42.12″N) and arrived at BPZ at the age of 2 yr 3.5 mo, with a body size of 866 kg (rhino A). The male rhinoceros was born at a zoo in Sioux Falls, SD (43°32′40.56″N) and arrived at BPZ at the age of 2 yr 2 mo, with a smaller overall body size, weighing 492 kg (rhino B). Because the animals arrived in winter, neither rhinoceros received outdoor access for the first few months at BPZ. When weather permitted, both animals were given outdoor access; however, rhino B was reluctant to go outdoors during his first year at BPZ. Seasonal weather fluctuations in the Midwest require the rhinoceros within this zoo to have limited outdoor access during cold winter months if ambient temperatures fall below 40°F (4°C). To prevent potential trauma, outdoor access is also limited if the outside yards are excessively icy or muddy.

The rhinoceros were each fed a diet of commercial pellets, mixed grass and alfalfa hay, and seasonally available fresh browse, with fruit and vegetables offered for training. The pelleted diets, their applicable dates fed, and manufacturer's labeled vitamin D_3 contents are listed in Table 1. Levels of vitamin D_2 were not listed by the manufacturer for the pelleted feed, and levels of vitamin D_2 provided by the hay fed to both animals were not evaluated, but unknown levels of vitamin D_2 could be assumed to be present in the feed provided. Pelleted diets were not independently tested for vitamin D_2 or D_3 content. The commercial pelleted diets offered were manufactured by Mazuri Feed (Purina Mills, St. Louis, MO 63144, USA) and HMS Zoo Diets Inc. (Bluffton, IN 46714, USA). Each rhino arrived at

the zoo on a different diet, and both were transitioned to the same diet as outlined in Table 1.

Sample collection and vitamin D analyses

Both rhinos were trained for voluntary blood collection for routine health monitoring by positive reinforcement methods within 60 days of arrival at the zoo. Unrestrained voluntary blood samples were collected (at monthly intervals if possible) from the medial radial vein with a 19-ga needle on a butterfly catheter (19 ga × ¾ inch × 12 inch SURFLO® winged infusion set, Terumo Corporation, 2-44-1 Hatagaya, Shibuya-ku, Tokyo 151-0072, Japan). From January 2013 through May 2016, 16 blood samples were successfully collected from rhino A and 24 samples from rhino B. Whole blood was placed into serum separator tubes (Integrated serum separator tubes, Covidien LLC, 15 Hampshire Street, Mansfield, MA 02048, USA) and centrifuged at 1,677 *g* for 5 min. Serum was removed and placed into red-top tubes (Monoject™ blood collection tubes, Covidien) for transfer on ice to Iowa State University Veterinary Diagnostic Laboratory (ISUVDL; Ames, IA). Samples were forwarded to Heartland Assays (Ames, IA) for determination of circulating 25OHD. For the purposes of this study, two additional banked serum samples from rhino B were submitted from frozen storage in the BPZ serum bank, which is maintained at –30°C, and submitted to ISUVDL and Heartland Assays for processing in May 2016. Blood samples were tested for 25OHD level using radioimmunoassay (RIA) standardized methods. The 25OHD assay consists of a two-step procedure.[13] The first procedure involves a rapid extraction of 25OHD and other hydroxylated metabolites from serum or plasma with acetonitrile. After extraction, the treated sample is then assayed using an equilibrium RIA procedure. The RIA method is based on an antibody that is cospecific for $25OHD_2$ and $25OHD_3$. The sample, antibody, and I^{125}-labeled $25OHD_3$ tracer are incubated for 120 min at 20–25°C. Phase separation is accomplished after a 20-min incubation at 20–25°C with a second antibody-precipitating complex. A nonspecific binding/addition buffer is added after this incubation before centrifugation to aid in reducing nonspecific binding. Total 25OHD concentration is calculated directly by counting I^{125} γ emission and comparing with a standard curve. The results are expressed in terms of 25OHD equivalents. To monitor assay performance, each assay includes an in-house control sample. The control is treated as an unknown specimen and multiple (total of five) determinations are made. The 25OHD assay has a range of 2.5–100 ng/ml and intra- and interassay CVs of 8.0 and 10.0.[17]

UVB data

The UV index is an international standard measurement of the strength of sunburn-producing UV radiation at a particular place and time. UV data were generalized from UV forecasts via the National Oceanic and Atmospheric Administration's UV Index Bulletins Archives.[30] Index data were used for Des Moines, IA beginning 01 January 2013 through 23 May 2016. Monthly averages were calculated and graphed using Microsoft Excel®. Sunlight exposure for each rhinoceros was logged each day by zoo staff on the basis of the time each individual stayed in the outdoor enclosures during daylight hours. Sunlight data were averaged to monthly levels by calculating time spent outside daily. Sunlight exposure was also calculated and graphed using Microsoft Excel. The only sunlight or UVB exposure that the rhinoceros received was during daylight hours in outdoor enclosures. Each outdoor yard has full sunlight exposure, with access to shade structures and mud wallows for both animals.

Statistical analyses

A genereal linear model (GLM) was used, with serum vitamin D as the dependent variable, sunlight exposure and dietary vitamin D as covariables, and individuum as a random factor; the residuals were inspected for normal distribution. Calculations were performed in SPSS 23.0 (IBM, Armonk, NY) with the significance level set to 0.05.

RESULTS

Early screening tests performed in January 2013 indicated that circulating 25OHD levels in both rhinoceros were substantially below published reference values of free-ranging rhino. Because of these low levels, an over-the-counter oral vitamin D_3 supplement (Hy-Vee Health Market All Natural Vitamin D_3-1000; 1,000-IU tablets, Hy-Vee Inc., West Des Moines, IA 50266, USA; dosed at 1,000 IU/kg feed; rhino A: 4,500 IU/day; rhino B: 2,500 IU/day) was offered from 07 March 2013 through 05 April 2013, in addition to D_3 already provided in the pelleted diets (Table 1). This supplement was only added during this

time period to determine if supplementation would raise circulating 25OHD to within normal reference levels.

The results for circulating 25OHD, sunlight exposure, environmental average UV level, and dietary vitamin D_3 are shown in Figures 1 and 2. Serum values of 25OHD increased for both animals from late spring to early fall each year (Fig. 1). Rhino A's 25OHD level seasonally increased notably in both 2013 and 2014. After 2014, rhino A ceased participation in blood collection training. For rhino A, 25OHD peaked at 26.1 ng/ml in 2013 and 40.3 ng/ml in 2014. In 2013, rhino B's reluctance to go outdoors was reflected in low serum 25OHD. These levels significantly increased in 2014 through 2016 as outdoor time increased. In 2013, rhino B's serum 25OHD levels peaked at 5.6 ng/ml, but increased to peaks of 30.6 ng/ml in 2014, 18.8 ng/ml in 2015, and 16.3 ng/ml by May of 2016. For both animals, circulating 25OHD levels dropped to less than 12 ng/ml each winter, with results of less than 2.5 ng/ml indicated as 0 ng/ml in these figures, as 2.5 ng/ml is the minimum detectable level of the RIA.

When levels of circulating 25OHD for both rhinos were compared with the monthly UV index (Fig. 1), there was a wave pattern that closely mirrored the seasonal fluctuations in UV, and therefore UVB levels. During the 3 yr of this study, the months of June, July, and August of each year had the highest monthly average UV index, which is expected for a location in the Northern Hemisphere. Each of these months averaged a UV level of at least 6.81, with multiple monthly summer UV averages of 7 and 8.

Similar to the wave pattern observed in the UV index, Figure 1 shows a yearly variation in circulating serum 25OHD level compared with sunlight (outdoor) exposure. Average daily sunlight exposures reflected the lack of time each rhinoceros spent outside in 2013. In 2013, rhino A averaged 221 min outside each day during September and no time outside during January or February. During 2013, rhino B spent even less time outside, with an average of just 75 min/day in September and no time outside during January or February. Over the next 2 yr, both rhinoceros spent more time in the outdoor enclosures, with most summer months averaging over 400 min each day of sunlight exposure. Cold winter weather conditions forced both rhinoceros indoors from December through March each year, with daily outdoor sunlight exposure times often less than 90 min/day.

Both rhinoceros received increases in daily vitamin D_3 intake in the spring of 2013, with additional oral supplementation of vitamin D_3 tablets. During this month-long trial, rhino A had a calculated daily D_3 intake total of 10,500 IU or 12 IU/kg of body weight (feed and supplement combined) and rhino B consumed up to 6,400 IU or 13 IU/kg of body weight each day. During the period of extra supplementation, rhino A's serum 25OHD level increased slightly from 4.5 to 7.5 ng/ml, but rhino B's serum level remained unquantifiable. Except for this limited supplementation, daily levels of feed D_3 remained relatively stable at levels between 4,500 and 6,250 IU (4–6 IU/kg of body weight) for each rhino, with minor fluctuations due to changes in quantity or type of pelleted ration fed.

In the GLM, sunlight exposure was highly significant ($F_{1,38} = 85.198$, $P < 0.001$), whereas diet was not ($F_{1,38} = 0.001$, $P = 0.978$). Individuum was a significant factor ($F_{1,38} = 20.853$, $P < 0.001$), indicating systematic differences in serum levels between the two animals. These findings are reflected in Figure 2 and indicate a strong association between UV exposure and 25OHD in both animals, and interindividual variation. These results may have been affected by the number of animals or number of samples available for this preliminary study.

DISCUSSION

This study investigated serum 25OHD in two eastern black rhinoceros held in captivity in a northern-latitude zoo. Over the course of this 3-yr period, the highest serum 25OHD level documented in either animal was 40.3 ng/ml, in July of 2014. This result is lower than the average of 55.7 ng/ml of $25OHD_3$ identified in 28 free-ranging black rhinoceros.[10]

During summer months, the animals in this study had 8 to 12 hr of sunlight exposure each day. It is theorized that the difference in circulating 25OHD levels between these two captive animals and the levels published for free-ranging black rhinoceros is due to differences in UV intensity at this latitude. The zoo in Des Moines, IA is located at 41°N, much farther from the equator and its stronger UVB rays than free-ranging black rhinoceros in their natural habitat (15°N to 34°S). Decreased exposure to UVB would result in less conversion of 7-DHC to previtamin D_3 and then to $25OHD_3$. Wide fluctuations in 25OHD were observed in both animals, coinciding with decreases in sunlight exposure times during indoor confinement due to management for seasonal

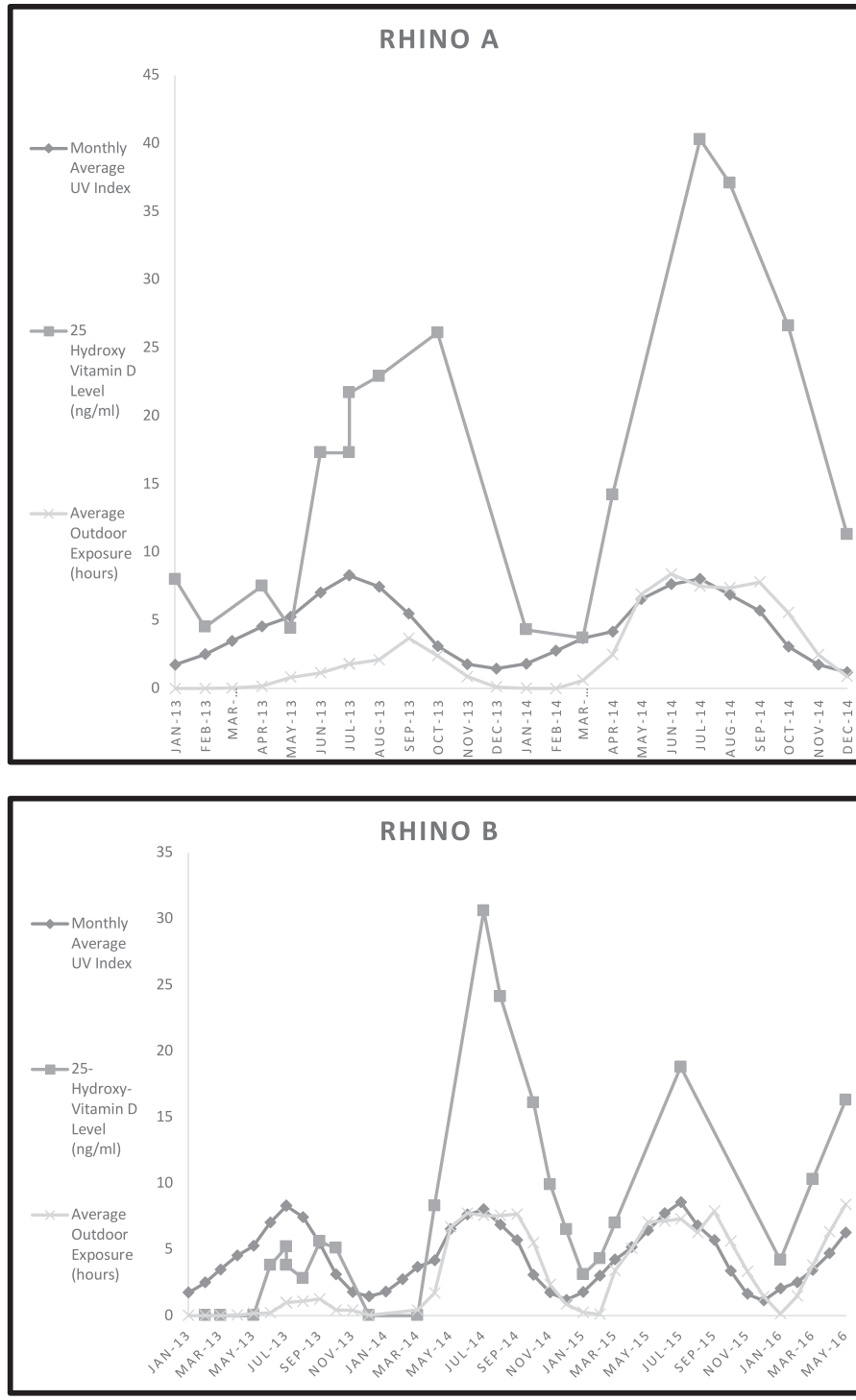

Figure 1. Blood serum levels of 25-hydroxy-vitamin D in two eastern black rhinoceros (*Diceros bicornis*) compared with average monthly ultraviolet index and average outdoor exposure times from January 2013 to May 2016.

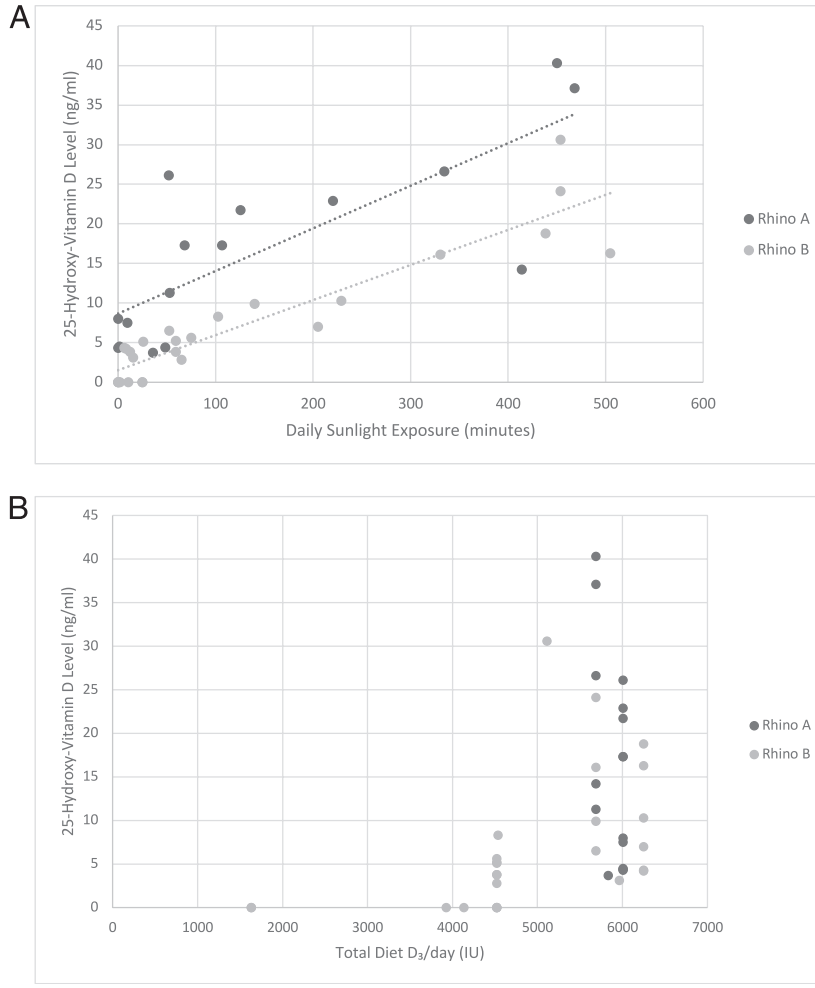

Figure 2. Correlations of (**a**) average monthly outdoor exposure and (**b**) vitamin D supplementation in feed with serum 25-hydroxy-vitamin D in two eastern black rhinoceros (*Diceros bicornis*).

weather conditions. Seasonal variation in 25OHD levels has been reported in other species, including humans, lemurs, and southern elephant seals living in northern climates.[19,32,33] The authors presume that free-ranging rhinoceros would be exposed to higher UVB throughout the year, and it would be likely that they would not show the large seasonal fluctuations in serum vitamin D levels reported in this study. The results of this study also indicate that although these captive rhinoceros were maintained at a relatively consistent level of supplemental vitamin D_3 in their diet (4,500–6,250 IU/day or 4–6 IU/kg of body weight), feed-based vitamin D_3 (and unknown levels of vitamin D_2) had very little effect on circulating 25OHD levels during winter.

In monogastric humans and pigs, and ruminants such as the domestic cow, low 25OHD concentrations are often accompanied by rickets during growth, or osteomalacia in adults.[26] For the two black rhinoceros in this study, there is currently no gross evidence of bone disease. Serum calcium and phosphorus concentrations were considered to be within normal reference ranges for the species at each time tested. Rhinoceros are hindgut fermenters, and in other hindgut fermenters such as the horse and rabbit, bone growth and calcium homeostasis can occur normally, independent of vitamin D.[6,9,11,14,23] Although vitamin D status of horses has been reported to be low relative to other species (4.7 to 22 nmol/L), and supplemental oral vitamin D has been reported to promote calcium and phosphorus absorption in horses, there are no reports of vitamin D deficiency to date in horses maintained in practical settings with some expo-

sure to sunlight.[23] In a study of domestic horses at pasture in New Zealand, between horses covered by standard horse blankets with neck rugs and unblanketed horses, there were no differences in either serum $25OHD_3$ or $25OHD_2$.[3] Differing from these domesticated hindgut fermenters, the black rhinoceros in this study do appear to produce marked levels of vitamin D in response to environmental UV exposure.

Beyond endocrine function in calcium homeostasis, current research suggests that circulating 25OHD concentrations are necessary to drive autocrine production of $1,25\text{-}(OH)_2D$ within a wide variety of cell types.[15,20,21,27] Autocrine production of $1,25\text{-}(OH)_2D$ has been shown to promote cell differentiation and apoptosis. Numerous epidemiological studies in humans demonstrate a link between low 25OHD status and various cancers, autoimmune disorders, abnormal cardiac function, and susceptibility to infections such as tuberculosis and sepsis.[2,7,15,20–22,27,28,31,34,35] A recent study of domestic foals in North America identified that vitamin D deficiency was highly prevalent in hospitalized foals, and that those with the lowest concentrations of $25(OH)D_3$ and $1,25(OH)2D_3$ had more severe disease and were more likely to die. These findings in hospitalized foals were similar to those reported in critically ill humans in which decreased concentrations of $25(OH)D_3$ have been associated with disease severity and outcome.[2,18]

It is possible that the comparatively lower level of circulating 25OHD in these two captive rhinoceros when there is no natural UVB available may contribute to various health complications. Black rhinoceros have been identified to have susceptibility to diseases such as *Mycobacterium tuberculosis* and certain types of neoplasia, including leiomyomas, squamous cell carcinomas, and cutaneous melanoma.[16,24] Tuberculosis, uterine leiomyomas, and other types of neoplasia have been associated with low (less than 30 ng/ml) 25OHD levels in humans.[4,5,8,20,25,28,30] Additionally, black rhinoceros-specific diseases with currently unexplained etiologies, including superficial necrolytic dermatopathy syndrome, idiopathic hemorrhagic vasculopathy syndrome, hemolytic anemia, and leukoencephalomalacia, may stem from immune system and neurodegenerative dysfunctions. Dysfunctions in immunocompetence, autoimmune disorders, and neurodegenerative disorders have also been linked to subclinical vitamin D deficiencies in humans.[8,27,28,35]

It should be noted that both captive rhinoceros in this study were apparently healthy at all time points for the duration of this study. Serum calcium levels remained within standard reference intervals for both animals when serum chemistry was performed. Ionized calcium was not performed for either animal. Additionally, the female rhinoceros (rhino A) became pregnant in the summer of 2015 and gave birth to a healthy live calf in October 2016, so individual effects on reproduction were not noted for these two individuals. However, because of their young age, long-term effects of seasonal hypovitaminosis D may not yet be evident. The authors hypothesize that the results of these two captive animals are most likely representative of a large number of black rhinoceros in human care, but additional testing is warranted to confirm this theory.

Because decisions to pursue diagnoses or initiate treatment are often based upon values falling outside reference intervals, the collection and analysis of reference values should be approached with diligence.[13] Because of the small number of animals in this study, additional research is necessary to document the relationship between latitude, dietary supplementation, and captive management strategies on circulating vitamin D levels in black rhinoceros compared with free-ranging animals in native habitats. Additional studies are also needed to correlate whether there are systemic health effects of subclinical hypovitaminosis D for animals in human care. This study would be strengthened by documenting ionized calcium levels at each time point for each animal, and evaluation of the complete diet (pellets and forage) and intake to determine actual consumed vitamin D_2 and D_3 levels. Very little is understood about the role vitamin D plays in the metabolism and immune function of the black rhinoceros. However, this study indicates that in these two captive animals, natural sunlight exposure seems to influence circulating 25OHD, and dietary supplementation in feed at current levels has little effect.

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