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RESEARCH ARTICLE

Evaluation of Season-Related Dietary Changes on the Serum Profiles of Fat-Soluble Vitamins, Mineral, Fatty Acids, and Lipids in the Captive Greater One-Horned Rhinoceros (*Rhinoceros unicornis*)

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Circulating concentrations of fat-soluble vitamins A, D, and E, minerals, fatty acids, and lipids were quantified in five captive greater one-horned rhinoceroses (*Rhinoceros unicornis*) throughout two time periods, during which two diets were offered. Animals were fed mixed-grass hay and concentrate pellets while managed in barns for winter housing (April sampling, winter diet). During the spring and summer, animals were fed the same amount of concentrate pellet but had free access to North American browse and grasses instead of dried forage (November sampling, summer diet). Levels of 25-hydroxyvitamin D and α-tocopherol were statistically higher in summer diet samples than in winter diet samples. Retinol was not statistically different between seasons, and β-carotene concentrations were undetectable at both time periods. Cholesterol, triglycerides, and non-esterified fatty acids were all significantly elevated following access to unlimited fresh forages in summer. Serum electrolytes were not different between the two time periods but differences in circulating minerals were noted (cobalt, inorganic iodine, and magnesium elevated in winter diet samples; selenium and zinc elevated in summer diet). Access to non-native fresh green forages resulted in improvement of several nutritional parameters in greater one-horned rhinoceroses, implying a benefit when fresh browse and access to grass is provided. Zoo Biol. 33:314–319, 2014.

Keywords: green forage; hay; health; herbivore nutrition; perissodactyla; reference range

INTRODUCTION

The greater one-horned (GOH) rhinoceros, classified as vulnerable by the IUCN [Talukdar et al., 2008], is native to regions of India and Nepal. Aggressive protection by authorities and support for conservation efforts have raised the free-ranging population from fewer than 200 earlier in the twentieth century to as many as 3,000 today [Talukdar et al., 2008]. Despite this potential success story, species recovery is not complete due to fragmented subpopulations, declining quality of habitat and continued poaching [Talukdar et al., 2008]. Maintaining this species in captivity is imperative to protect against its extinction, to continue to increase public awareness of conservation actions, for education, and for research. Although this species is widely distributed throughout zoos and aquariums, management and husbandry can vary, resulting in health concerns and variable breeding success [Dierenfeld, 1999; Dierenfeld and Traber, 1992; Jones, 1979].

The GOH rhinoceros is classified as an intermediate browser due to 70–89% of its free-ranging diet consisting of grasses [Laurie, 1982; Pradhan et al., 2008]. In the wild, GOH rhinoceroses have been found to eat over 180 different plant species, with availability greatly affected by location, seasonal variation in weather, and activity of other animals, including humans [Laurie, 1982]. Captive diets do not aim to reflect this variation in intake, but are designed to be

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nutritionally balanced and provide adequate energy, protein, vitamins, and minerals, taking into account the age, sex, and reproductive status of the individual to satisfy the animals' physiological requirements [Dierenfeld, 1993]. Most captive diets consist of combinations of mixed hay and straw, pressed hay, concentrate pellets, produce, and fresh forages (grasses, branches, and leaves) [Von Houwald, 2002]. Captive recommendations to offer browses such that nutrient intake more closely matches natural forage consumption have been suggested, but concerns include acquiring appropriate quantities, and seasonal and nutritional variability in composition [Clauss et al., 2002; Dierenfeld, 1995; Dierenfeld et al., 1994, 2005, 2006].

Circulating fat-soluble vitamin levels (vitamins A and E) have been measured in captive and free-ranging black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceroses, and captive Sumatran (*Dicerorhinus sumatrensis*) and GOH (*Rhinoceros unicornis*) rhinoceroses. These investigations have primarily been triggered by incidences of several disease syndromes in captive rhinoceroses linked with low vitamin status [Clauss et al., 2002; Dennis et al., 2007; Ghebremeskel, Lewis, and Du Toit, 1988; Jones, 1979; Von Houwald and Flach, 1998]. Serum vitamin E levels were found to vary significantly between captive and free-ranging black rhinoceroses, and subsequent dietary studies revealed that forages in captivity supply lower amounts of vitamin E, and require supplementation to maintain serum levels comparable to free-ranging individuals [Dierenfeld et al., 1990, Dierenfeld, Du Toit, and Braselton, 1995; Ghebremeskel and Williams, 1988; Ghebremeskel, Lewis, and Du Toit, 1988]. Captive GOH rhinoceroses were found to have significantly lower vitamin A levels (measured as all-*trans*-retinol) than captive black and white rhinoceroses, and lower vitamin E levels than captive white rhinoceroses [Clauss et al., 2002]. Circulating 25-hydroxyvitamin D was found to be higher in free-ranging individuals compared with captive animals in zoological facilities, which is attributed to the role of natural sunlight in the synthesis of vitamin D and its metabolites [Clauss et al., 2002; Savolainen et al., 1980]. These differences highlight the importance of knowing species-specific reference ranges, correlated with feeding strategies/dietary management, in order to monitor health status and ensure adequate fulfillment of nutritional requirements. Serum and plasma mineral concentrations have also been published for four rhinoceros species, and analysis supports the use of equine references ranges for comparison with rhinoceros species for the majority of minerals, excluding perhaps selenium, molybdenum, and zinc [Dierenfeld et al., 2005].

Green forage was found to be an important source of carotenes and tocopherols in horses, and contained a higher concentration of both compared with hay [Ahlswede and Konermann, 1980; Garton, Vandernoot, and Fonnesbeck, 1964; Maenpää, Koskinen, and Koskinen, 1988]. Carotenes and tocopherols are oxidized readily, and their concentrations in hay may decrease during storage, with forage losing up to 90% of vitamin E activity during maturation and senescence [Schryver and Hintz, 1983; Zintzen, 1972]. Seasonal variations have been noted in both domestic horse serum carotene and α-tocopherol levels, thought to be secondary to changes in forage composition and dietary intake [Garton, Vandernoot, and Fonnesbeck, 1964; Maenpää, Koskinen, and Koskinen, 1988]. Dietary analyses of forages consumed by free-ranging and captive black rhinoceros suggested that forages utilized in captivity supply lower amounts of vitamin E than native diets [Dierenfeld et al., 1990, Dierenfeld, Du Toit, and Braselton, 1995], and that captive diets can only attain comparable levels to native forage when supplemented [Ghebremeskel et al., 1991]. Circulating vitamin E concentrations in ungulates also vary with the polyunsaturated fatty acid (PUFA) content of forages consumed [Dierenfeld, 1989; Dierenfeld and Traber, 1992]. This is inferred from studies with domestic livestock in which the ingestion of freshly emerging plants containing much higher levels of PUFAs is associated with depletion of body stores of vitamin E [Dierenfeld, 1989; Dierenfeld and Traber, 1992]. Therefore, as absorption of vitamin E is commensurate with lipid uptake, ensuring functional fat digestion through the measurement of serum lipids and fatty acids is prudent in understanding the metabolism of vitamin E in hoofstock [Dierenfeld, 1999].

The Wilds managed a herd of two sub-adult and three adult GOH rhinoceros, which were maintained in a 400-acre pasture during the spring and summer, and housed in barns during the fall and winter months. Diet consisted of a commercial pelleted ration throughout the year, with ad libitum mixed-grass hay in fall and winter, and ad libitum fresh grass and browse while managed in pasture. Given previous research based on the nutritional content of fresh forages versus hay, it was hypothesized that there would be a difference in the serum profiles of fat-soluble vitamins, minerals, lipids, and fatty acids during the two management periods. The goal of this study was to directly compare the serum profiles of fat-soluble vitamins, minerals, fatty acids, and lipids from animals fed fresh forage versus grass hay, in order to assess the importance of fresh forages to the nutritional management of captive GOH rhinoceroses.

MATERIALS AND METHODS

In 2011, five GOH rhinoceroses were housed at "the Wilds," a conservation facility in southeastern Ohio. One adult male (8 years of age), two adult females (11 and 15 years of age), and two sub-adult females ($<$4 years of age) were managed during the spring and summer on a 400-acre pasture with continuous access to fresh Ohio grasses (tall fescue (*Festuca arundinacea*), birdsfoot trefoil (*Lotus corniculatus*), smooth brome (*Bromus inermis*), orchardgrass (*Dactylis glomerata*), sweet clover (*Melilotus officinalis*), and ragweed (*Ambrosia* sp.) and other plants. Animals spent fall and winter months housed in barns, with minimal and intermittent access to fresh forage, and were fed ad libitum mixed-grass hay (tall fescue, birdsfoot trefoil, smooth brome, orchard grass, sweet clover ragweed). A locally formulated pellet was fed throughout the year, with the same amount (5 kg) offered to each individual during both seasons (Faler Feed Store, Inc., Lithopolis, OH). Each individual was sedated in both April (winter diet) and November 2011 (summer diet) to perform ophthalmic and reproductive examinations, and to have blood collected to assess general health and nutritional status. April blood samples were collected to reflect serum values following barn housing for approximately five months, and before any access to fresh forages. November values reflected pasture management (7-month period), during which they were offered negligible grass hay.

The skin over the medial radial vein was cleaned with an iodophor scrub and rinsed with alcohol prior to the aseptic collection of 50 milliliters of blood. Sample handling was consistent between groups, including protecting samples from sunlight and exposure to excessive air to reduce oxidation. Two milliliters of whole blood were preserved in ethylenediaminetetraacetic acid for hematological testing, while the remainder was placed in plain serum tubes on ice packs until centrifugation for serum separation (within 1 hr of collection). Whole blood and fresh serum were submitted for complete blood count and serum biochemistry to IDEXX Laboratories (Westbrook, ME). Serum was stored in cryovials at −80°C until assessment (Diagnostic Center for Population and Animal Health, Michigan State University [MSU], Lansing, MI). Samples from each time period were analyzed simultaneously following standard protocols (MSU; www.animalhealth.msu.edu). Non-esterified fatty acid, electrolyte, cholesterol, and triglyceride values were determined using an Olympus chemical analyzer (Center Valley, PA). Serum was prepared for fatty acid analysis using methyl-esterification, and analyzed by gas chromatography (MSU). Serum was analyzed for retinol, β-carotene (assay lower limit of detection 0.05 μg/ml), and α-tocopherol by high performance liquid chromatography (MSU), and 25-hydroxyvitamin D by radioimmunoassay (MSU). Trace minerals and inorganic iodine levels were determined using inductively coupled plasma mass-spectroscopy (MSU). Parameters from each time period were compared using one-tailed Wilcoxon signed-rank test at 95% significance level (IBM SPSS version 22.0.0, Armonk, NY).

RESULTS

The fat-soluble vitamins measured in this study are presented in Table 1; electrolytes and mineral values are found in Table 2; and lipids and fatty acids profiles in Table 3.

TABLE 1. Serum fat-soluble vitamins (mean ± SD) measured from five greater one-horned rhinoceroses (*Rhinoceros unicornis*) during two sampling periods reflecting different dietary forage sources

Nutritional parameter measured	Winter diet (Barn housing)	Summer diet (Pasture management)	*P*-value	Clauss et al. [2002], *R. unicornis*-specific data
25-hydroxyvitamin D (nmol/L)	22.4 ± 2.93	32.8 ± 7.44	**0.031**	–
Retinol (μg/ml)	0.035 ± 0.005	0.041 ± 0.008	0.063	0.03 ± 0.02
β-carotene (μg/ml)	<0.050	<0.050	–	–
α-tocopherol (μg/ml)	0.400 ± 0.021	0.764 ± 0.137	**0.031**	0.34 ± 0.36

Statistically significant *P*-values in bold font.

TABLE 2. Serum electrolytes and minerals (mean ± SD) measured from five greater one-horned rhinoceros (*Rhinoceros unicornis*) during two sampling periods reflecting different dietary forage sources

Nutritional parameter measured	Winter diet (Barn housing)	Summer diet (Pasture management)	*P*-value	Dierenfeld et al. [2005], *R. unicornis*-specific data
Calcium (mg/dl)	11.40 ± 0.39	11.60 ± 0.26	0.063	12.07 ± 2.01
Chloride (mmol/L)	92.00 ± 2.19	90.40 ± 1.36	0.125	–
Cobalt (ng/ml)	0.866 ± 0.223	0.478 ± 0.096	**0.031**	–
Copper (μg/ml)	2.530 ± 0.117	2.490 ± 0.253	0.219	2.00 ± 0.49
Inorganic iodine (ng/ml)	35.7 ± 22.4	16.90 ± 8.89	**0.031**	–
Iron (μg/dl)	152.2 ± 34.2	129.2 ± 22.4	0.094	130 ± 19
Magnesium (mg/dl)	2.660 ± 0.162	2.06 ± 0.08	**0.031**	1.73 ± 0.47
Molybdenum (ng/ml)	10.90 ± 1.60	9.10 ± 1.07	0.063	5.75 ± 2.04
Manganese (ng/ml)	0.86 ± 0.25	1.94 ± 1.17	0.063	1.89 ± 0.26
Phosphorus (mg/dl)	4.04 ± 0.74	3.96 ± 0.17	0.375	4.07 ± 0.25
Potassium (mmol/L)	4.14 ± 0.30	3.76 ± 0.26	0.063	4.14 (no SD)
Selenium (ng/ml)	77.00 ± 4.34	90.00 ± 3.52	**0.031**	98.5 ± 2.1
Sodium (mmol/L)	133.20 ± 1.17	134.80 ± 1.17	0.063	133.0 ± 9.6
Zinc (μg/ml)	1.190 ± 0.173	1.790 ± 0.103	**0.031**	2.35 ± 1.07

Statistically significant *P*-values in bold font.

TABLE 3. Serum lipid and fatty acids (mean ± SD) measured from five greater one-horned rhinoceroses (*Rhinoceros unicornis*) during two sampling periods reflecting different dietary forage sources

Nutritional parameter measured	Winter diet (Barn housing)	Summer diet (Pasture management)	*P*-value
Cholesterol (mg/dl)	51.40 ± 6.09	69.40 ± 8.73	**0.031**
Triglyceride (mg/dl)	14.30 ± 2.62	21.40 ± 4.41	**0.031**
Non-esterified fatty acids (mEq/L)	0.17 ± 0.11	0.722 ± 0.150	**0.031**
Lauric acid C12:0 (%)	3.48 ± 0.98	2.17 ± 0.43	**0.031**
Myristic acid C14:0 (%)	4.45 ± 1.65	3.35 ± 0.45	0.094
C14:1n5c (%)	3.23 ± 2.01	1.92 ± 1.80	0.156
Palmitic acid C16:0 (%)	15.00 ± 1.96	15.60 ± 1.70	0.500
C16:1n7c (%)	1.96 ± 0.96	1.69 ± 0.18	0.313
Stearic acid C18:0 (%)	6.99 ± 1.65	6.46 ± 0.86	0.156
C18:1n7c (%)	2.10 ± 0.66	1.27 ± 0.07	0.063
C18:1n9c (%)	13.60 ± 3.54	17.20 ± 1.35	**0.031**
C18:2n6c (%)	44.80 ± 6.04	46.40 ± 3.46	0.406
C20:4n6c (%)	1.57 ± 0.44	1.47 ± 0.81	0.406
C22:1n9c (%)	2.77 ± 1.51	2.47 ± 0.37	0.500

Statistically significant *P*-values in bold font.

Complete blood counts and biochemistry values were within the normal range reported for this species in captivity and animals were considered healthy [Flesness, 2002]. A significant difference was noted between diets in alkaline phosphatase (ALP, mean ± SD, winter diet 108 ± 22.2 U/L, summer diet 158.6 ± 61.2 U/L, $P = 0.031$), and aspartate aminotransferase (AST, mean ± SD, winter diet 59 ± 9.19 U/L, summer diet 75.4 ± 17.1 U/L, $P = 0.031$).

Vitamin D, measured as its abundant metabolite 25-hydroxyvitamin D, was found to differ significantly between April and November, with animals coming in from pasture management showing statistically higher vitamin D levels. Mean retinol was numerically, but not statistically higher after pasture management. β-carotene levels were not measurable at either time period. Mean serum α-tocopherol was 91% higher following summer diet (0.764 μg/ml) than winter diet (0.400 μg/ml).

Electrolyte values from both winter diet and summer diet blood samples were within reference ranges utilized [ISIS, Dierenfeld et al., 2005]. Multiple mineral differences were noted when values were compared to a previously published reference range in GOH rhinoceroses [Dierenfeld et al., 2005]. Serum cobalt, inorganic iodine, magnesium, selenium, and zinc differed between the two time periods (Table 2). Electrolytes showed no difference between the two time periods but several minerals were found to be statistically different. Cholesterol, triglycerides, non-esterified fatty acids, and fatty acid C18:1n9c were all statistically higher in November (summer diet), with lauric acid being significantly lower in April (winter diet). No other differences were noted in the fatty acid profile between the two time periods.

DISCUSSION

All animals were considered to be healthy based on their complete blood counts and serum biochemistry panel values, and no changes to routine management practices were made in order to perform this comparative study. This report appears to provide the first report of vitamin D concentrations in a GOH rhinoceros (free-ranging or captive). The most abundant metabolite 25-hydroxyvitamin D was found to be significantly higher during November sampling (summer diet) than during barn housing. The synthesis of vitamin D by the skin is negligible when sunlight is limited [Savolainen et al., 1980], and sun-cured hay is likely to contain minimal amounts of vitamin D_2 [Maenpää, Lappeteläinen, and Virkkunen, 1987]. Access to unfiltered sunlight is limited during winter months due to cold environmental temperatures restricting access to outside paddocks. This is in contrast to pasture management where continuous access to sunlight during daylight hours likely results in elevated vitamin D levels during the summer diet-sampling period. This is similar to findings in the black rhinoceros, in which free-ranging animals had higher levels than captive individuals [Clauss et al., 2002]. Vitamin D levels in free-ranging GOH rhinoceroses may be elevated compared with levels reported here due to the variation in latitude (26.14–26.75°N, compared with 39.94°N in this study). In comparison, seasonal differences in 25-hydroxyvitamin D were not noted in captive African elephants (*Loxodonta africana*), although this could have been related to southern US location and daily outdoor exposure time [Miller et al., 2009].

Circulating retinol values from both April and November were similar to values noted by Clauss et al. [2002] (0.03 ± 0.02 μg/ml) from ten captive individuals. Mean retinol tended to be higher during pasture management than when housed in barns, which is expected since fresh green forage contains more carotenes (which are converted into retinol in the intestines) than stored feeds [Ahlswede and Konermann, 1980]. However, no significant differences were found between the two time periods, suggesting that serum retinol concentrations are not useful indicators of vitamin A status in this species [Maenpää, Koskinen, and

Koskinen, 1988]. Circulating carotenoid β-carotene was not detected in the 10 serum samples evaluated. This is similar to findings reported by Slifka et al. [1999], and Clauss et al. [2002], in which captive black rhinoceroses had negligible to low circulating carotenoids despite moderate carotenoid levels in their diet. The exact mechanism for the lack of measurable β-carotene has not been determined but hypotheses include differences in the selective absorption process between species [Furr and Clark, 1997]. Further studies are required to determine if GOH rhinoceroses are similar to black rhinoceroses in their metabolism and absorption of carotenoids since captive exotic herbivores are expected to have adequate amounts of β-carotene and lutein in their diet [Slifka et al., 1999].

Serum concentrations of α-tocopherol are correlated with those of serum lipids, since vitamin E is transported in plasma lipids [Maenpää, Koskinen, and Koskinen, 1988]. Serum α-tocopherol levels were adequate for both seasons when compared to an existing reference range from 10 captive individuals [Clauss et al., 2002]. However, given that serum total cholesterol was also adequate in the winter diet samples, lower serum α-tocopherol at this time compared with pasture management is likely a result of reduced amounts of ingested vitamin E. Rhinoceros species appear to have a complete absence of plasma high-density lipoproteins (HDL) and the only lipoproteins detected were characteristic of low-density lipoproteins (LDL) [Leat et al., 1979]. This is unlike other perissodactyls, such as equids and tapirs, in which both HDL and LDL were found [Leat et al., 1979].

The domestic horse has been found to be an excellent physiological model for mineral metabolism in rhinoceros species, including GOH [Clauss et al., 2005]. Zinc deficiency has been proposed to be involved in the etiology of chronic pododermatitis in captive GOH rhinoceros, and therefore roughage-based diets may require supplementation [Clauss et al., 2005]. The current study demonstrated statistically higher circulating zinc levels when animals were fed a fresh forage-based diet versus dry roughage, even when the level of concentrate (the primary source of zinc) offered remained the same, suggesting that animals may benefit from access to fresh forage in order to increase dietary zinc intake or bioavailability. Other minerals that differed between the time periods were cobalt (required in the formation of vitamin B-12), selenium (required to prevent nutritional muscular dystrophy), magnesium (which plays an important role in muscle biochemistry), and inorganic iodine (which is essential to thyroid function). Hay fed to animals at the Wilds is grown on-site, and given the history of surface mining at the Wilds during the 1940s to 1980s, it is not surprising to have found significant differences in several minerals, due to the effects of soil erosion and reclamation activity on the land.

Lipids and non-esterified fatty acids were all significantly elevated following pasture management, which is expected due to the loss of lipid and fatty acid concentration in stored forage due to the oxidative processes that occur when plants are cut [Grant, Brown, and Dierenfeld, 2002]. Two fatty acids demonstrated differences; lauric acid was significantly higher during barn management, and oleic acid (C18:1n9c) was higher following access to fresh forages. Fats are noted to be a major component of free-ranging black rhinoceros browses and therefore, access to North American browse and grasses are the likely reason for the elevated fat levels when fed the summer diet [Dierenfeld, 1993]. Oleic acid is a PUFA that is principally sourced from vegetable and plant fats [Goodnight et al., 1982]; therefore, an elevation in this fatty acid in the summer diet blood values is not unexpected since overall fat levels also went up following pasture management.

Serum ALP appeared to be higher in summer diet samples, similar to domestic horse studies in which ALP levels were elevated during summer months and declined during winter housing [Maenpää, Koskinen, and Koskinen, 1988; Maenpää, Lappeteläinen, and Virkkunen, 1987]. Factors such as a more restrictive environment during stall housing compared with pasture, reducing mechanical stress on limbs, a change in nutrition, and an alteration of the hormonal balance have all been hypothesized as causes and are applicable to this situation [Maenpää, Koskinen, and Koskinen, 1988; Maenpää, Lappeteläinen, and Virkkunen, 1987]. Reduced muscle activity may also be responsible for the reduced serum AST during barn housing, as both hepatocellular trauma and myopathies will cause elevations in this enzyme [Peak, 2003].

CONCLUSIONS

1. Access to non-native fresh green forages resulted in improved nutritional parameters in GOH rhinoceroses.
2. Levels of 25-hydroxyvitamin D and α-tocopherol were increased following prolonged access to natural sunlight and ingestion of green forages, respectively.
3. Multiple mineral differences were noted between the two time periods (cobalt, inorganic iodine and magnesium higher on winter diet, selenium and zinc higher on summer diet), which may be reflective of varying bioavailability in the two forages evaluated (fresh green forage and stored hay). Electrolyte levels were not affected by seasonal diet change.
4. Circulating cholesterol, triglycerides, non-esterified fatty acids, and the fatty acid C18:1n9C were higher in GOH rhinoceros following access to unlimited fresh forage compared with ad libitum dry hay intakes.
5. Overall, superior nutritional value from fresh green forage versus dried forage has been demonstrated in this study, through the elevation of multiple nutritional parameters.

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