CONTINUING ASSESSMENT OF VITAMIN ANALYSIS RELIABILITY ACROSS LABORATORIES: EXAMPLES IN WHITE AND BLACK RHINO SPECIES (*CERATOTHERIUM SIMUM* AND *DICEROS BICORNIS*)

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Abstract

As part of an ongoing assessment of vitamin E supplementation in white and black rhino species (2017–2018) at Disney's Animal Kingdom, the first 6 months of assessment utilized 2 laboratories (A and B) for vitamin E analysis in split serum samples, and 2 laboratories (C and D) for vitamin E analysis in split fecal samples. In both species (n = 3, each), fecal samples were taken every 2 weeks. Blood samples were taken every 2 weeks for black rhinos and every month for white rhinos. Fecal samples had similar vitamin E concentrations when compared between labs C and D (n = 45; r = 0.67; $P < 0.01$). However, there were major differences seen in serum vitamin E levels between labs A (1.3 ± 0.5 µg/mL) and B (0.7 ± 0.9 µg/mL), which had previously been utilized for a vitamin E study in elephants (Sullivan *et al*., 2016). There was no discernable correlation between the split serum samples (n = 46; r = 0.14; P = 0.36). A possible cause of the vitamin E laboratory discrepancy was that Lab B was performing extra dilutions on serum samples, as their stated minimum sample volume was actually a minimum volume requiring dilution, not for analysis, hence raising the minimum detection limit. This was a key communication lesson, as some of our collection animals are able to readily provide a slightly larger quantity of blood through routine collection procedures. Due to continuing questions, minimal clinical samples have been sent to Lab B for the immediate future. Lab A, while reliable, may not be open to high frequencies of clinical samples, so may not be a viable option to use long term. We continue to work to find a commercial laboratory that can be relied on for consistent serum vitamin analysis, as well as having the ability to do mineral analyses. This is critical for correct nutritional assessment and supplementation plans for sensitive exotic species such as rhinos.

Introduction

Assessment of the impact of nutrition on the health of exotic animals ideally includes clinical serum profiles including vitamin and mineral panels. However, when these are obtained as part of a yearly clinical assessment, trust in the laboratory values is essential. Diet changes are often made at Disney's Animal Kingdom based on values of vitamins or minerals in serum. One of the most common vitamins supplemented is vitamin E (□-tocopherol). Serum vitamin E concentrations have been reported to be lower in managed black and white rhinoceros when compared with free-ranging rhinos, irrespective of age and sex (Clauss *et al*., 2002; Dierenfeld, 1994). Only bilary excretion of vitamin E has been demonstrated as a route of exit from mammalian bodies, when vitamin E exceeds absorptive capacity, primarily through tocopherol transport protein in the liver

(Combs and McClung, 2016). Due to historical issues when studying vitamin E in the serum and feces of elephants, multiple labs were utilized in a similarly designed ongoing assessment study of vitamin E in white and black rhinoceros at Disney's Animal Kingdom (Sullivan *et al*., 2016).

Methods

White and black rhinos at Disney's Animal Kingdom (white: 1.2 adults; black: 2.1 adults) were assessed for vitamin E status in serum and fecal output as part of a 1-year study testing differential dosing. For the purposes of this abstract, the effect of dosing on serum and fecal vitamin levels will not be presented, but rather a comparison of split samples for each individual across time in terms of correlation. Two labs were utilized (referred to as Lab A and Lab B) for serum assessment of vitamin E as □-tocopherol. Blood samples were obtained in royal blue top vacutainers, left at room temperature for 1 hour, manually agitated, and spun down at 3500 rpm for 10 minutes. Serum was placed into cryovials and frozen at -80°C prior for a minimum of 24 hours. Serum samples were shipped monthly to laboratories on the same day. Feces were obtained as fresh sample ($\leq$ 30 minutes from defecation), flash frozen with liquid nitrogen on site, and maintained at -20°C until a monthly same day laboratory shipment. Two labs were utilized (referred to as Lab C and Lab D) for flash frozen fecal assessment of vitamin E as □-tocopherol. In both species, fecal samples are taken every 2 weeks, with blood samples taken every 2 weeks in black rhinos and once monthly in white rhinos. Control serum samples obtained from a large phlebotomy collection in a black rhino were kept at -80C, and at least one was sent to Lab A and B with every shipment. Comparisons were performed using Pearson's *r* and bivariate correlation comparisons in IBM SPSS Statistics 22 (Armonk, NY). Alpha was considered significant at $P < 0.05$.

Results

The samples considered for each lab comparison varied with $n = 49$ (fecal comparisons), and $n = 46$ (serum comparisons). There were 23 white rhino and 26 black rhino fecal samples split and analyzed. White rhino fecal samples had similar ranges on a dry matter basis between Lab C (465 ± 166 mg/kg vitamin E) and Lab D (316 ± 164 mg/kg vitamin E). Black rhino fecal samples had overlapping ranges on a dry matter basis between Lab C (1260 ± 407 mg/kg vitamin E) and Lab D (726 ± 453 mg/kg vitamin E). These split rhino samples were positively and significantly correlated for feces ($r = 0.67$; $P < 0.01$; Figure 1). There were 16 white rhino and 30 black rhino serum samples split and analyzed. Overall, the average serum vitamin E in µg/mL was found to be 1.3 ± 0.5 for Lab A and 0.7 ± 0.9 for Lab B. The split rhino samples were not correlated, nor significant for serum ($r = 0.14$; $P = 0.36$; Figure 2).

Discussion

The inconsistencies of high performance liquid chromatography analysis of vitamin E across laboratories may be due to differences in sample handling procedures, equipment calibration, and, of course, the possibility of human error. While citing a standard HPLC procedure, laboratories producing variable and inconsistent results, both between and within themselves is a long standing challenge (Greaves *et al*., 2014). In our small comparison, we found troubling lack of patterns in reliability in one lab. An entire month of serum samples was found to be below detection limits for Lab B but relatively high for Lab A; however, the control samples sent were consistent for Lab A only. Investigation into this inconsistency revealed that Lab B's minimum amount of serum requested was only enough to dilute out, rather than run straight, raising the detection limit. Rather than request more sample, the lab reported the values as undetectable. This one incidence led to

continued questions on reliability of this laboratory for this assay and questions on historical low values seen as well. If methodologies are not completely proprietary, they should be compared systematically moving forward. While fecal analysis appeared far more consistent across laboratories, some variation did occur, especially in the last month's analysis where temperature during shipment may have been a factor, despite both going overnight on ice. Lab D did have a delayed arrival that month, in hot temperatures, perhaps contributing to a variation. Prior to the month of May's samples results were $r = 0.89$ with $P < 0.001$. However, control fecal samples (extra doubles of at least one sample every shipment) were consistent for both labs C and D, indicating greater reliability of these labs.

Despite methodological similarities between laboratories there appear to be confounding factors inhibiting uniform reporting and standardization of vitamin assays (Greaves *et al*., 2014). Sending controls across time, no matter what medium of sample is sent, can be critical to assessment of and confidence in commercial laboratories. Standardizing this quality control procedure is a necessary investment to ensure proper diet supplementation for animal health.

Acknowledgements
The authors would like to thank the Savannah and Ituri teams at Disney's Animal Kingdom for their aid in collections, as well as the many Nutrition team interns that also contributed.

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