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INVESTIGATION OF FACTORS POTENTIALLY ASSOCIATED WITH SERUM FERRITIN CONCENTRATIONS IN THE BLACK RHINOCEROS (*DICEROS BICORNIS*) USING A VALIDATED RHINOCEROS-SPECIFIC ASSAY

Jessye Wojtusik, M.S., Ph.D., and Terri L. Roth, M.S., Ph.D.

Abstract: Iron overload disorder (IOD) can lead to organ dysfunction and may exacerbate other diseases in the critically endangered black rhinoceros (*Diceros bicornis*). It is important to develop methods for monitoring the progression of iron storage (hemosiderosis), diagnosing the disease, and evaluating treatments in this species. Traditionally, an equine enzyme immunoassay (EIA) was used to measure rhinoceros ferritin, a serum protein correlated to iron stores. The goal of this study was to validate a rhinoceros-specific assay and investigate factors potentially associated with ferritin concentrations in black rhinoceros. A ferritin EIA developed for Sumatran rhinoceros was validated for black rhinoceros via Western blot analysis of liver ferritin and confirmed parallelism of serum samples to the EIA standard curve and used to analyze serum samples ($n = 943$) collected from 36 black rhinoceros (<1–33 yr) at 14 U.S. institutions. Mean (±SEM) serum ferritin concentration was 6,738 ± 518 ng/ml (range: 85–168,451 ng/ml). Concentrations differed among individuals with eastern black rhinoceros (7,444 ± 1,130 ng/ml) having a higher mean ferritin than southern black rhinoceros (6,317 ± 505 ng/ml; $P < 0.05$) and higher mean values in wild-born (11,110 ± 1,111 ng/ml) than captive-born individuals (3,487 ± 293 ng/ml; $P < 0.05$). Ferritin concentrations did not differ between young rhinoceros (<5 yr old; 2,163 ± 254 ng/ml) and adults (7,623 ± 610 ng/ml) and were not correlated with age ($r = 0.143$) or time in captivity ($r = 0.146$, wild born; $r = 0.104$, all animals). Ferritin concentration was not impacted by sex (female: 2,086 ± 190 ng/ml; male: 8,684 ± 717 ng/ml), date, month, or season of collection ($P > 0.05$). Data indicate ferritin concentrations are variable and not necessarily associated with IOD; ferritin is not recommended for diagnosing or monitoring IOD in black rhinoceros.

Key words: Black rhinoceros, enzyme immunoassay, ferritin, hemochromatosis, hemosiderosis, iron overload disorder.

INTRODUCTION

Iron is a fundamental element in biological systems, crucial for oxygen binding and transport throughout the body; however, there is no direct mechanism for iron excretion, and therefore homeostasis is sustained through controlled absorption.[2] Excess iron is stored intracellularly, typically within the liver or spleen, as focal deposits of hemosiderin; this process is known as hemosiderosis. Iron overload disorder (IOD), known to impact a variety of species, including primates,[17] birds,[37] horses,[24] tapir,[7] red deer,[32] and three species of rhinoceros—African black (*Diceros bicornis*),[35] Sumatran (*Dicerorhinus sumatrensis*),[36] and Indian (*Rhinoceros unicornis*)[3]—results in excessive iron storage (severe hemosiderosis). Excessive hemosiderin deposition results in pathological changes and subsequent visceral damage, a disease termed "hemochromatosis" in humans. As clinical signs of IOD and hemochromatosis in rhinoceros are difficult to interpret, diagnosis generally occurs postmortem through histological analysis of tissue samples.

Morbidity, associated with excessive iron storage, is caused primarily by accumulation in the liver, heart, and endocrine organs, particularly the pancreas, pituitary, or gonads, that eventually limits their function.[4,8] In humans, hemochromatosis is associated with cirrhosis of the liver,[27] diabetes,[9] cardiomyopathy,[18] and various other maladies.[1] There is speculation that IOD exacerbates other diseases known to afflict black rhinoceros, including hemolytic anemia, mucocutaneous ulcer disorder, and leukoencephalomalacia.[33,34] Histological examination of various tissues collected from black rhinoceros postmortem revealed increased hemosiderin deposition in captive animals as compared to wild individuals, and severity of deposition seemed to be associated with time in captivity.[22] Additionally, Smith et al[39] found mild to moderate positive correlations

From the Center for Conservation and Research of Endangered Wildlife, Cincinnati Zoo and Botanical Garden, Cincinnati, Ohio 45220, USA (Wojtusik, Roth). Correspondence should be directed to: Dr. Wojtusik (jessye.wojtusik@cincinnatizoo.org).

between common stored iron measures and both age and time in captivity in black rhinoceros. IOD has been documented in Sumatran rhinoceros,[36] and despite low reported values for stored iron measures, it is evident that Indian rhinoceros are also susceptible.[3,31] Frequent large-volume phlebotomies have been used to mitigate disease progression with some anecdotal success;[29,44] however, such treatments are logistically challenging and limited in long-term feasibility. Recent development of an oral iron chelator for use in humans may offer hope for treating wildlife;[8] however, it is difficult to diagnose the disease state in rhinoceros. Current techniques for monitoring iron accumulation often involve tracking serum ferritin concentrations, but its accuracy in diagnosing IOD or hemochromatosis has been questioned.[3,4,36]

Ferritin is an acute phase globular protein complex responsible for binding iron for storage. Serum ferritin is often used as an indicator of total iron stores, as concentrations are correlated with stored iron levels in some species,[40,41] and high concentrations can indicate excessive stored iron.[21] However, serum ferritin concentrations were not correlated to liver iron stores of the Sumatran rhinoceros,[36] and "normal" ferritin values were reported in an Indian rhinoceros a month prior to finding severe liver iron accumulation postmortem.[3]

The gene coding for ferritin is highly conserved; however, the ferritin protein may vary among species.[46] Smith et al[39] demonstrated that an equine ferritin antibody would cross-react to bind rhinoceros ferritin; therefore, a ferritin enzyme immunoassay (EIA) developed for horses has traditionally been used to measure ferritin in rhinoceros serum samples. However, there have been some discrepancies in results. Shook et al[38] compared black rhinoceros serum ferritin concentrations using two assays, both developed for measuring equine ferritin, but each employed different standards (equine or black rhinoceros ferritin); values were correlated, but those generated using the equine standard were twice the concentration of those generated using the rhinoceros standard. In addition, Roth et al[36] noted serum ferritin values that did not align with health status of individual Sumatran rhinoceros, and values differed when the same samples were evaluated repeatedly using the horse ferritin assay. Due to these inconsistencies, usefulness of the equine ferritin assay for monitoring iron storage and/or diagnosing IOD was questioned, and a rhinoceros-specific ferritin EIA was developed using a monoclonal antibody against Sumatran rhinoceros liver tissue ferritin.[36] The results from that study demonstrated that serum ferritin concentration is not a reliable biomarker of IOD progression and disease in Sumatran rhinoceros.[36]

If ferritin is an accurate measure of iron deposition, patterns of expression should be predictable, consistently changing over time (with age)[1,39,49] and in response to factors known to impact iron stores, especially when an individual is afflicted by IOD. In humans, women have consistently lower ferritin concentrations than men, likely due to blood loss during menses, as ferritin levels increase in postmenopausal women.[49] In addition, it is evident that ferritin concentrations may be influenced by the reproductive status of a female, possibly due to the use of iron in fetal and placental formation.[16,36] Dietary changes due to seasonal or geographical availability of certain foodstuffs may influence iron intake and thus ferritin concentrations.[19] Therefore, as iron availability may differ by location, individuals and/or subspecies[28] may have developed different physiological responses to iron uptake, thereby impacting ferritin levels. IOD has not yet been confirmed in wild individuals; however, those born in the wild and brought into captivity appear to be at risk of developing hemosiderosis,[22] though it is unclear if these individuals are at a greater or lesser risk compared to their captive-born counterparts.

The goal of this study was to validate the rhinoceros-specific ferritin assay for measuring black rhinoceros serum ferritin and to determine if differences in ferritin concentrations are associated with several factors, including age, season, sex, subspecies, and location of birth, and to evaluate the efficacy of this assay as a tool for diagnosing IOD and monitoring its progression. Hypotheses included: 1) the antibody developed for the Sumatran rhinoceros ferritin EIA would bind serum ferritin of black rhinoceros, making the EIA a useful tool for measuring ferritin concentrations of black rhinoceros; 2) several factors other than IOD influence ferritin concentrations; and 3) ferritin is not a reliable method for diagnosing or monitoring IOD in the black rhinoceros.

MATERIALS AND METHODS

Animals and sample collection

Serum samples ($n = 943$; 3–237 samples/individual) collected during 1990–2016 from 36 black rhinoceros ranging in age from <1 to 32 yr and

Table 1. Mean ferritin values (±SEM) and range for black rhinoceros based on sex, subspecies, birth, location, and age-group.

	No. of individuals	No. of samples	Mean ferritin ± SEM (ng/ml)	Range (ng/ml)
Sex				
Female	11	282	2,086 ± 190	138–23,493
Male	25	661	8,684 ± 717	85–168,451
Subspecies[a]				
Southern	18	611	6,317 ± 505	138–89,710
Eastern	18	332	7,444 ± 1,130	85–168,451
Birth location[a]				
Wild born	7	400	11,110 ± 1,111	138–168,451
Captive born	29	543	3,487 ± 293	85–46,432
Age-group				
Young	17	156	2,163 ± 254	140–25,549
Adult[b]	27	787	7,623 ± 610	85–168,451
Total	36	943	6,738 ± 518	85–168,451

[a] P-value < 0.05.
[b] Includes senior animals.

maintained at 14 U.S. institutions were analyzed using the rhinoceros ferritin EIA (Table 1). Two individuals died while participating in the study, one due to unknown causes; the other was euthanized, and IOD was confirmed. All samples were banked for other purposes and used opportunistically in this study with permission from each owning institution. Samples were stored at –20°C and/or –80°C and experienced one to two freeze/thaw cycles prior to use in this study. Ferritin remains stable at temperatures up to 80°C, and concentrations remain constant through multiple days to weeks at room temperature (RT) and through at least six freeze/thaw cycles.[6,14,23,42,43] Due to the extreme stability of the ferritin protein, concern over storage length and/or method is limited. Sampling frequency varied by individual; however, each month and season were represented within the data set. Liver tissue samples were collected postmortem and stored at –20°C.

Ferritin isolation

Ferritin was isolated from black rhinoceros liver using methodologies previously described and used to isolate ferritin from Sumatran rhinoceros liver.[10,13,36] Briefly, 1 g of liver tissue in 4 ml of sterile water was homogenized at 8,000 *g* for 4 min on ice. Samples were transferred to a 75°C water bath for 15 min and agitated continuously for the first 3 min and then for 1 min of every remaining 3 min. Samples were placed on ice for 30 min followed by centrifugation at 16,000 *g* for 20 min. Supernatant was collected, treated with a final concentration of 35% saturated ammonium sulfate, and incubated approximately 15 hr at 4°C. Samples were then centrifuged at 2,600 *g* for 20 min, the supernatant was removed, and the remaining pellet was reconstituted in 2.75 ml of phosphate-buffered saline (PBS) per gram of liver. The suspension was centrifuged at 20,000 *g* for 30 min, and the supernatant was filtered through 100-μM Amicon centrifugal filters (EMD Millipore, Billerica, Massachusetts 01821, USA) via centrifugation at 4,500 *g* for 15 min. Remaining concentrate (that which did not pass through filter) was diluted in 3.5 ml of PBS and subject to centrifugation using the same filter a second time. The concentrate above the filter was diluted with PBS up to 0.5 ml and frozen at –80°C in aliquots of 25 μl. Protein concentration was determined using a Bradford assay with purified equine spleen ferritin as the standard (Sigma Aldrich, St. Louis, Missouri 63103, USA).

Gel electrophoresis and immunoblotting

Black rhinoceros liver ferritin (BRF) was subjected to gel electrophoresis using purified equine spleen ferritin and Sumatran rhinoceros ferritin[36] as positive controls to verify sample purity as well as white rhinoceros ferritin to demonstrate specificity to rhinoceros ferritin. Native Mark (Life Technologies, Carlsbad, California 92008, USA) was used as a molecular weight ladder. Each sample (25 μg) was diluted 1:1 with Laemelli sample buffer (Bio Rad, Hercules, California 94547, USA) and then brought up to a volume of 20 μl with PBS and loaded in duplicate into the lanes of a 3–15% tris-acetate gel (0.2 M tris-acetate buffer, pH 7; Jule Biotechnologies, Inc.,

Milford, Connecticut 06460, USA) and electrophoresed at ~75 V for 15 min and then 95 V for approximately 1.5 h. The gel was cut into halves (each containing a ladder and duplicate of each sample). One-half of the gel was stained with potassium ferricyanide (1.317 g + 20 ml dH_20 + 20 ml HCl; Sigma Aldrich) to confirm that isolated protein was positive for iron.[25] The second half of the gel was transferred onto a polyvinylidene fluoride (Bio Rad) membrane for use in immunoblotting. The membrane was blocked for 1 hr at RT in blocking buffer (5% fat-free dry skim milk powder; Saco Foods, Middleton, Wisconsin 53562, USA) in PBS. The membranes were then continuously agitated in 1:1,000 monoclonal indicator antibody 1D6.A3[36] in blocking buffer with 0.1% Tween 20. Membranes were washed for 5 min (3×) in PBS + 0.1% Tween (PBST) and then incubated in 1:10,000 goat anti-mouse antibody (AbCam, Cambridge, Massachusetts 02139, USA) in blocking buffer for 1 hr at RT. Membranes were washed for 5 min (3×) in PBST and incubated in 4 ml of chemiluminescence (optiblot ECL max detect kit; AbCam) and imaged using an Odyssey imaging system (LI-COR Biotechnology, Lincoln, Nebraska 68504, USA).

Ferritin enzyme immunoassay

Serum ferritin concentrations were measured with the rhinoceros-specific ferritin sandwich enzyme immunoassay (EIA) previously developed and validated for Sumatran rhinoceros, described in detail by Roth et al.[36] Serum samples (neat to 1:200 dilution), standards (47–3,000 ng/ml BRF), and quality controls (200 and 800 ng/ml BRF; quality control) were run in duplicate and used as the lower and upper limits, respectively, for the range of accepted sample values. Any sample falling outside of this range was rerun at a dilution that ensured that the value fell within range. HRP-ID6 was used at a concentration of 470 ng/ml.

Statistical analysis

Data were analyzed using a linear mixed model with repeated measures and correlation analysis. A saturated model was created with the following factors included: individual, subspecies, location, sex, age, age-group, collection date (month, year, season), captive/wild status of individual at birth, and time in captivity. This analysis controls for varying sample sizes among individuals in addition to the other listed factors. Ferritin concentration was specified as the main effect. Seasons were defined based on the approximate solstices as follows: winter, January–March; spring, April–June; summer, July–September; and fall, October–December. Animals were classified by age: those 5 yr old or younger at the time of collection were deemed "young," those older than 5 yr and younger than 25 yr as adult, and those older than 25 yr as senior.

Insignificant factors that accounted for the least variation in the model (collection date, location, age) were removed from the final analysis. When testing for a correlation between time in captivity and ferritin concentrations, wild-born individuals were considered in one analysis, and then all individuals were included in a second; for animals born in captivity, time was calculated from date of birth. Necropsy and pathology reports were available for a select few individuals, and timing and cause of death were considered during the interpretation of changes in ferritin concentrations over time. For comparative purposes, all data are presented as arithmetic means[28,36,38] ± SEM, and all statistical tests were completed using SPSS for Windows (Version 24; IBM Corp., Armonk, NY 10504, USA).

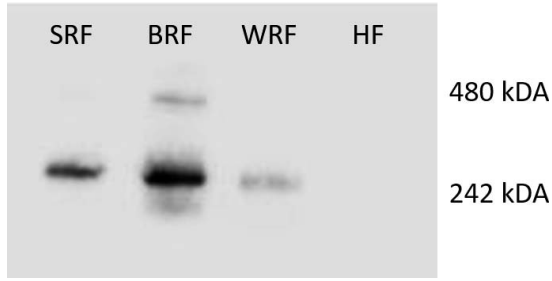

Figure 1. Western blot verifying binding of indicator antibody 1D6.A3 to the primary band (240 kDA) of Sumatran (SRF), black (BRF), and white (WRF) rhinoceros liver ferritin but not horse ferritin (HF) and at the apoferritin band (480 kDA) of black rhinoceros.

RESULTS

Western blot analysis verified binding of indicator antibody 1D6.A3 to BRF (at 240 and 480 kDA). The antibody did not bind to horse liver ferritin but did bind ferritin isolated from the livers of Sumatran, black, and white rhinoceros, demonstrating the antibody's specificity for rhinoceros ferritin (Fig. 1). Staining of gels with potassium ferricyanide revealed the presence of iron in the bands of extracted ferritin (data not shown). Serially diluted BRF and serum samples

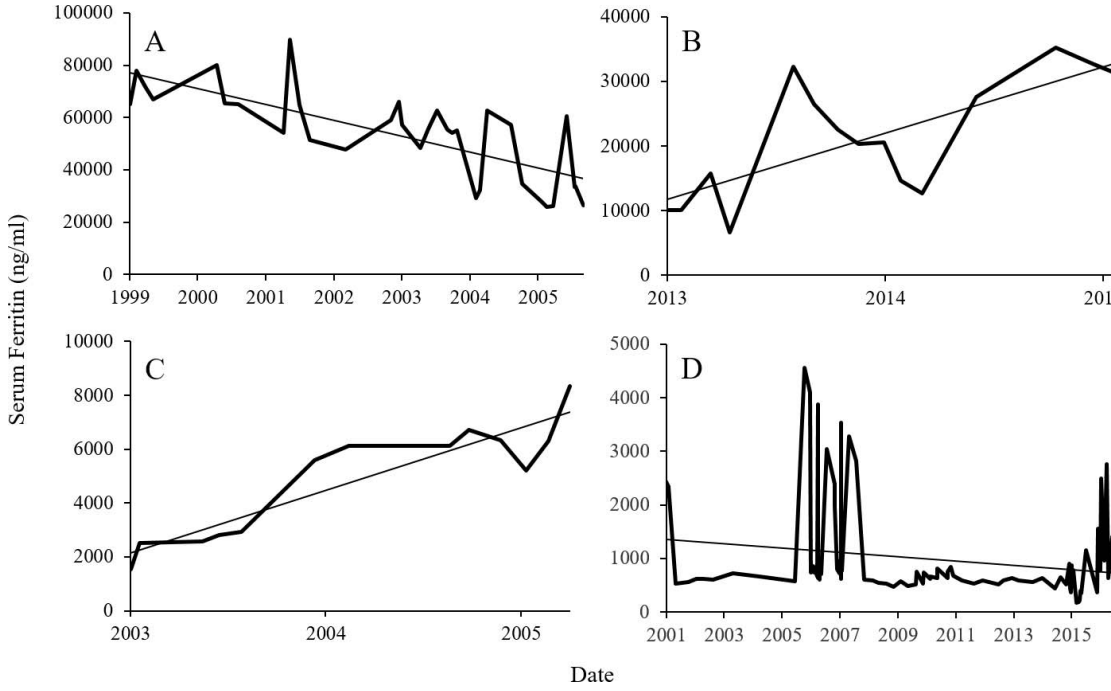

Figure 2. Serum ferritin concentrations of select black rhinoceros individuals, plotted over time with trend lines. **A.** Individual #7. Animal died within a month of last sample collection; cause of death unknown. **B.** Individual #30. At time of publication, animal is alive and not displaying any signs of illness. **C.** Individual #8. Animal died from *Mycobacterium tuberculosis* with evidence of hemosiderosis 8 yr after last sample collection. **D.** Individual #19. Animal developed clinical signs of IOD (confirmed postmortem) prior to rise in ferritin at end of life.

(neat up to dilution of 1:128) displayed curves parallel to Sumatran rhinoceros liver ferritin ($r^2 = 0.998$ and 0.991, respectively), demonstrating validity of the assay. The inter- and intra-assay coefficients of variation were maintained at less than 15% and 10%, respectively.

Mean serum ferritin concentration was 6,738 ± 518 ng/ml, with a value range of 85–168,451 ng/ml across the population (Table 1) and an individual mean range of 547–98,336 ng/ml (data not shown). Collection date did not significantly impact serum ferritin concentrations regardless of classification (month, year, or season). Serum ferritin concentrations did not differ between adult and senior animals, so these animals were combined into one group, termed "adult," for further analysis. Variation among individuals was significant ($P < 0.05$; Fig. 2), and due to this variation, what appeared to be differences in means of age-group and sex were not statistically different. There was no difference between young (2,163 ± 254 ng/ml) and adult groups (7,623 ± 610 ng/ml; $P > 0.05$; Table 1) and no meaningful correlation ($r = 0.143$) between age and ferritin concentration (Fig. 3). Similarly, time in captivity was not strongly correlated with serum ferritin values ($r = 0.146$ for wild-born individuals; $r = 0.104$ for all individuals) and was not included in the final model. Serum ferritin concentrations did not differ between females (2,086 ± 190 ng/ml) and males (8,684 ± 717 ng/ml; $P > 0.05$; Table 1). Ferritin concentrations were greater in eastern black rhinoceros (7,444 ± 1,130 ng/ml) than the southern subspecies (6,317 ± 505 ng/ml; $P < 0.05$) and in wild-born individuals (11,110 ± 1,111 ng/ml) as compared to captive born (3,487 ± 293 ng/ml; $P < 0.05$).

Longitudinal profiles for each individual were created (selected rhinoceros profiles are shown in Fig. 2). Two individuals (#7, Fig. 2A, and #19, Fig. 2D) died during sampling. Of the three individuals (#7, #30, and #35) displaying the highest ferritin values on average, #7 (Fig. 2A) died at the age of 18 yr due to unknown causes, #30 (Fig. 2B) is alive and clinically healthy (at the time of publication), and #35, the individual with the highest ferritin values (data not shown), was euthanized at the age of 36 yr due to arthritis,

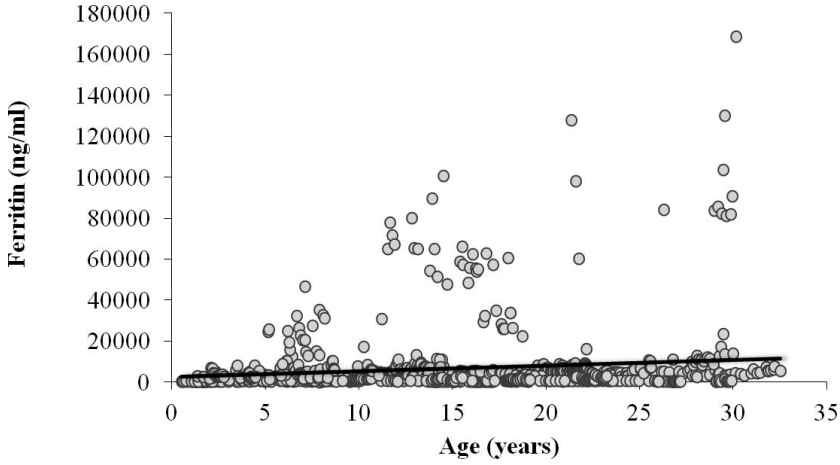

Figure 3. Ferritin concentrations of all black rhinoceros individuals (gray circles), plotted by age of animal, including trend line (black).

though hemosiderosis was mentioned in the necropsy report. Additionally, individual #8 (Fig. 2C), which experienced increasing ferritin levels over the course of ~3 yr, only died 8 yr after the last sample collection at 33 yr of age due to *Mycobacterium tuberculosis*. Although hemosiderosis was noted at necropsy, it was not reported as severe. Serum samples from the only individual known to have died with IOD, #19 (Fig. 2D), contained <1,000 ng/ml of ferritin, well below the species' average, prior to signs of illness. Ferritin concentrations in some samples (but not all) were elevated in the months leading up to death but remained below average and were lower than values in several samples collected approximately 10 yr earlier.

DISCUSSION

This study is the largest comparative analysis of serum ferritin in captive black rhinoceros and potentially the only study to analyze longitudinal rhinoceros ferritin data collected via serial sampling of individuals over the course of many years. The results offer unique insight into the variability of ferritin over time and among and within individuals and suggest that ferritin is not an accurate biomarker of IOD in black rhinoceros. Furthermore, several other factors appeared to be correlated with serum ferritin concentrations, making data interpretation in the context of IOD challenging.

The rhinoceros-specific assay developed by Roth et al[36] was validated for use with black rhinoceros serum by two different methods. Prior to assay validation, gel electrophoreses followed by protein and iron-specific staining were used to prove that the isolated black rhinoceros liver protein was ferritin. The protein was then subjected to Western blot analysis. Because Roth et al[36] demonstrated that the capture antibody (5D10.B5) was not specific, binding both equine and rhinoceros ferritin, only the highly specific indicator antibody (1D6.A3), which did not bind to horse ferritin, was tested. 1D6.A3 proved to cross-react well with black rhinoceros ferritin. The second validation was confirmed parallelism between the standard curve of serially diluted black rhinoceros serum and those of both black and Sumatran rhinoceros liver ferritin. Interestingly, although the monoclonal antibodies used in the assay were generated against Sumatran rhinoceros liver ferritin,[36] ferritin concentrations detected in black rhinoceros serum often exceeded (sometimes substantially) those reported for Sumatran rhinoceros, thereby providing additional evidence of high cross-reactivity with black rhinoceros ferritin.

Ferritin values varied significantly among and within individuals, with a range of 85 to 168,451 ng/ml, and the standard deviation of 15,814 ng/ml further demonstrates the extent of the variability among samples and individuals. Interestingly, these mean values are much higher than those reported for Sumatran rhinoceros but do not differ from those generated from the equine assay: 7,160 ± 9,784 ng/ml (SD) for adult black rhinoceros.[35] Regardless of assay choice, individual variation makes it difficult to generate a threshold value that could represent the transition from "normal" values to abnormal values of the

disease state. Many rhinoceros in this study exhibited ferritin values equal to or lower than those reported for wild individuals,[28] though the overall means tended to be greater. Generally, mean concentrations reported for wild individuals are often much lower than those reported for captive individuals;[28,35,38] therefore, it is postulated that all values reported for captive individuals are "abnormal." Two individuals were determined to be outliers; however, as each consistently experienced high values across 34 and 10 samples, respectively, they were included in the final analysis. Smith et al[39] and Miller et al[28] reported data with values considered higher than most, and the authors believe it is important to include these data in the final analysis, as high values are not uncommon and are important to consider when reflecting on the implications of data. Additionally, a separate analysis excluding these individuals demonstrated similar results to the final model and did not impact major conclusions.

Unfortunately, high values did not necessarily indicate that the animal was at risk of developing IOD, similar to what was reported by Roth et al[36] in Sumatran rhinoceros. Individuals #30 and #35 had the greatest mean ferritin concentrations, and at the time of publication, #30 is showing no obvious signs of illness. Individual #35 was euthanized at 36 yr of age due to arthritis, and though there was hemosiderosis noted at necropsy, neither IOD nor hemochromatosis was diagnosed, suggesting that the extreme ferritin values were associated with arthritis or something else entirely. Furthermore, individual #8 experienced rising ferritin values over the course of 3 yr and lived for another 8 yr before succumbing to *M. tuberculosis*. Hemosiderosis was noted postmortem; however, it is unclear if ferritin values were rising 8 yr prior to death in response to iron storage, other disease, or another physiological condition. These findings were not entirely surprising since a study in humans revealed that increased ferritin levels are more likely to be associated with obesity, fatty liver, or diseases other than hemochromatosis.[47]

In the case of individual #19 specifically, ferritin was low during much of her 15-yr sampling period and did not increase prior to her illness and signs of IOD, which was later confirmed following her death. Ferritin concentrations did rise prior to death but only after clinical signs were noted. Additionally, these values remained below the species' average and were lower than some samples collected 10 yr prior. Furthermore, this animal developed pneumonia during her illness, which could have been the stimulus for elevated ferritin toward the end of her life. This case demonstrates that ferritin is not a reliable early diagnostic measure of IOD.

Ferritin concentrations did not differ between age-groups (young versus adult), similar to wild black rhinoceros,[28] and, as reported for Sumatran rhinoceros,[36] ferritin was not found to be correlated with age ($r = 0.143$; Fig. 3) or with time in captivity, regardless of whether captive-born individuals were included in the analysis or not ($r =$ 0.104 and 0.146, respectively). Smith et al[39] demonstrated a mild correlation of ferritin with time in captivity ($r = 0.43$; $n = 32$). The discrepancy in data between this study and the study conducted by Smith et al is likely due to differences in sample number and strategy (one sample per animal vs. longitudinal sample collection). Serial sampling conducted during this study allowed for a more comprehensive and accurate view of how ferritin changes over time in individuals.

Similar to results reported for wild individuals,[12,28] ferritin concentrations were not impacted by sex of the animal. This differs from reports in humans that describe higher values for men than women;[49] however, as this difference is likely in response to blood loss due to menses, it is not applicable to rhinoceros. Unfortunately, there were not enough samples collected from pregnant individuals to determine if concentrations were impacted by reproductive status as noted in Sumatran rhinoceros and humans.[16,36] It is speculated that due to differences in diet type (ie, browsers versus grazers), bioavailability of iron, and amount of iron-binding compounds within the diet, mechanisms regulating iron absorption evolved differently for rhinoceros species.[5,19] Helary et al[19] found varying levels of iron in rhinoceros diets dependent on location, and differing ferritin levels have been reported for black rhinoceros inhabiting different regions.[28] If iron deposition was mediating ferritin levels, physiological adaptations to ranges with varied iron bioavailability might explain subspecies differences. However, eastern black rhinoceros and southern black rhinoceros ranges overlap, so it seems unlikely that a difference in range or bioavailable iron is the primary factor responsible for the subspecies difference revealed in this study. Surprisingly, wild-born individuals had significantly higher levels of ferritin than those born in captivity. This result was unexpected, as ferritin values are generally lower in wild versus captive individuals.[22,28,38] It is possible that captive-born individuals may have a developmental

adaptation mitigating the physiological response of ferritin. Alternatively, the wild-born sample size was relatively small ($n = 7$) and included the two individuals with the highest mean ferritin concentrations, likely skewing this result.

Serum ferritin is often considered a reliable marker of stored iron,[11] as levels closely parallel iron reserves in some species, such as horses, pigs, and humans.[40,41,45] However, ferritin is an acute phase protein; serum concentrations rise during instances of infection, inflammation, diet change, metabolic syndromes, and many other diseases not specific to iron storage.[1,20,28,30,38,47] Additionally, it is important to note that the strong relationship between serum ferritin and iron storage is often dependent on the status of the individual being "healthy" and "normal."[4,11,20,26,45] It is possible that serum ferritin in rhinoceros rises in response to IOD; however, it is now apparent that there are other stimuli for ferritin production, possibly associated with any one of the aforementioned physiological states, that may mask any increase in ferritin that may occur in association with iron load. Furthermore, because high ferritin concentrations are associated with such a wide variety of illnesses and physiological states, relying on it as a biomarker of IOD could lead to misdiagnosis.[15,16] Support for this argument is demonstrated by the extremely high ferritin concentrations in many individuals showing no obvious signs of illness demonstrated in this and previous studies. In Sumatran rhinoceros, liver iron concentrations did not correlate with ferritin, and ferritin was not indicative of disease progression.[36] Furthermore, as seen with individual #19 in this study, ferritin concentrations were extremely low months prior to the development of illness due to IOD. Black rhinoceros serum ferritin concentrations are variable, high concentrations are not predictive of clinical illness, and low concentrations can exist in sick individuals with extensive hemosiderosis; therefore, ferritin is not a useful tool for diagnosing IOD or monitoring its progression in the African black rhinoceros. It is imperative that future IOD research include developing new reliable methods to monitor progression of hemosiderosis, diagnose the disease state, and evaluate the efficacy of new treatments.

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