SERUM FERRITIN CONCENTRATION IS NOT A RELIABLE BIOMARKER OF IRON OVERLOAD DISORDER PROGRESSION OR HEMOCHROMATOSIS IN THE SUMATRAN RHINOCEROS (*DICERORHINUS SUMATRENSIS*)

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Terri L. Roth, M.S., Ph.D., Paul R. Reinhart, and Jennifer L. Kroll, R.V.T., A.A.S.

Abstract: The aim of this study was to determine if ferritin is a reliable biomarker of iron overload disorder (IOD) progression and hemochromatosis in the Sumatran rhinoceros (*Dicerorhinus sumatrensis*) by developing a species-specific ferritin assay and testing historically banked samples collected from rhinos that did and did not die of hemochromatosis. Ferritin extracted from Sumatran rhino liver tissue was used to generate antibodies for the Enzyme Immunoassay. Historically banked Sumatran rhino serum samples ($n = 298$) obtained from six rhinos in US zoos ($n = 290$); five rhinos at the Sumatran Rhino Conservation Centre in Sungai Dusun, Malaysia ($n = 5$); and two rhinos in Sabah, Malaysia ($n = 3$) were analyzed for ferritin concentrations. Across all US zoo samples, serum ferritin concentrations ranged from 348 to 7,071 ng/ml, with individual means ranging from 1,267 ($n = 25$) to 2,604 ng/ml ($n = 36$). The ferritin profiles were dynamic, and all rhinos exhibited spikes in ferritin above baseline during the sampling period. The rhino with the highest mean ferritin concentration did not die of hemochromatosis and exhibited only mild hemosiderosis postmortem. A reproductive female exhibited decreases and increases in serum ferritin concurrent with pregnant and nonpregnant states, respectively. Mean (±SD) serum ferritin concentration for Sumatran rhinos in Malaysia was high (4,904 ± 4,828 ng/ml) compared to that for US zoo rhinos (1,835 ± 495 ng/ml). However, those in Sabah had lower ferritin concentrations (1,025 ± 52.7 ng/ml) compared to those in Sungai Dusun (6,456 ± 4,941 ng/ml). In conclusion, Sumatran rhino serum ferritin concentrations are dynamic, and increases often are not associated with illness or hemochromatosis. Neither a specific pattern nor the individual's overall mean ferritin concentration can be used to accurately assess IOD progression or diagnose hemochromatosis in this rhino species.

Key words: Enzyme immunoassay, ferritin, hemochromatosis, hemosiderosis, iron overload, rhinoceros.

INTRODUCTION

Iron overload disorder (IOD; the excessive storage of iron in organ tissues) affects a wide variety of wildlife species maintained in captivity including many birds,[30,36] primates,[9,27] rodents,[16] marine mammals,[37,51] tapirs,[8] bats,[25] and two of the four rhino species, the African black rhinoceros (*Diceros bicornis*)[31,39,46] and the Sumatran rhinoceros (*Dicerorhinus sumatrensis*).[39] Monitoring the progression of this disorder and identifying when it has developed into hemochromatosis, the diseased state in which organ function is compromised by hemosiderin, has been challenging because of restricted handling and sampling of such species and the paucity of normal, healthy data for comparison. Therefore, assessments of the severity of hemosiderosis and diagnosis of hemochromatosis typically occur only postmortem.

Although hemochromatosis is infrequently reported as the primary cause of death in captive black rhinos, it has proven lethal to two Sumatran rhinos in the past decade, and hemosiderosis has been noted in multiple organs of most African black and Sumatran rhinos at necropsy.[39] Furthermore, 20% of black rhino deaths are due to infectious diseases, including salmonella and tuberculosis,[42] both of which have demonstrated enhanced virulence in iron-rich environments.[26,32] In contrast, captive white rhinos (*Ceratotherium simum simum*) are rarely lost to infectious disease and median longevity is 34.2 yr,[24] almost twice that for black rhinos (17.8 yr).[40] In light of these statistics, there is much speculation about the potential role of IOD in the health and longevity of the two browsing rhinos.

Because hemochromatosis afflicts humans, there is abundant research on the topic, some of which may be applicable to wildlife. In humans, ferritin is the primary intracellular iron-binding protein found in organ tissues, and only low extracellular concentrations are typically found

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in circulation. Because a direct association between body iron stores and serum ferritin concentrations has been reported in several species, including humans,[35] horses,[48] pigs,[47] cats,[2] and dogs,[52] serum ferritin is often used to diagnose iron overload disease. However, ferritin is not specific to this disease: it is an acute-phase protein that can increase in response to many inflammatory reactions, including chronic infections and neoplastic diseases.[33]

Ferritin seems ubiquitous among living organisms, occurring in plants, algae, and bacteria in addition to the animal kingdom, and the genes are highly conserved among species.[49] However, the protein can be immunologically species-specific,[41] such that antibodies generated against ferritin of a specific species may bind poorly, or not at all, to the ferritin of another species.[46,52] Furthermore, although ferritin is consistently associated with iron, its regulatory mechanisms and functional repertoire across the wide variety of taxa vary and have not been fully elucidated in wildlife species.

Efforts to monitor IOD in living rhinos have relied primarily upon measures of serum ferritin concentrations and transferrin saturation. Rhino ferritin antibodies have not been available to date, but antibodies to equine ferritin were found to cross-react to African black and white rhino ferritin[46] and have been used with either rhino or horse ferritin serving as the standard.[45,46] Assay results have led to the conclusions that ferritin concentrations are high in virtually all captive black and Sumatran rhinos,[39] increase rapidly after capture,[31] and worsen with age and time in captivity.[14,39,46] However, when the same equine assay was used to analyze Sumatran rhino serum, inconsistencies in serum ferritin concentrations were noted and values did not always coincide with the physiological state of the animal. For example, serum ferritin concentrations for a Sumatran rhino that died of hemochromatosis were no different than those for an old Sumatran rhino that died of other causes years later.[44]

The aim of this study was to determine if ferritin is a reliable biomarker of IOD progression and hemochromatosis in Sumatran rhinos by developing a species-specific ferritin assay and testing historically banked samples collected from rhinos that did and did not die of hemochromatosis. The two hypotheses being tested were that 1) Sumatran rhino serum ferritin concentrations determined in a rhino ferritin-specific assay will differ from those produced by an equine based assay and 2) serum ferritin concentrations assessed by a rhino-specific assay can be used to monitor the progression of IOD to hemochromatosis in Sumatran rhinos.

MATERIALS AND METHODS

Rhino ferritin extraction and purification

Ferritin was extracted from Sumatran rhino liver tissue previously collected postmortem and stored frozen. The extraction protocol generally followed previously published procedures[17,23] with a few minor modifications. Ultrafiltration tubes (Amicon 100 μM Ultra 4, Millipore, Fisher Scientific, Waltham, Massachusetts 02451, USA) were used to concentrate and desalt the final sample two times using phosphate-buffered saline (PBS) as the diluent. Final protein concentration was determined using a Bradford assay, and the sample was stored at −80°C.

Validation of ferritin sample purity

To confirm the sample contained ferritin and no other proteins, the sample was subjected to non-denaturing electrophoresis through a 3–8% tris-acetate protein gel (Nupage, Novex, EA0375, Life Technologies, Carlsbad, California 92008, USA) using purified equine spleen ferritin (F4503, Sigma-Aldrich, St Louis, Missouri 63103, USA) as a positive control and Native Mark (Life Technologies) as the standard ladder. The gel was stained with Coomassie blue to identify the protein bands and to match the pattern with that of the equine ferritin. A second gel was stained with potassium ferricyanide (Sigma-Aldrich) to confirm the presence of iron within the protein.[34]

Monoclonal antibody production

An aliquot of the isolated Sumatran rhino ferritin (SRF) was sent frozen to the Bioexpression and Fermentation Facility, Department of Biochemistry and Molecular Biology, Davison Life Sciences, University of Georgia (Athens, Georgia 30602, USA) for monoclonal antibody production in mice. After preliminary tests of hybridoma supernatants, two IgG monoclonal antibodies were chosen for Enzyme Immunoassay (EIA) development: 5D10.B5 (IgG2bK) and 1D6.A3 (IgG1k).

EIA development and assay protocol

The sandwich EIA capture antibody (5D10.B5) was stored in a −80°C freezer. The indicator antibody (1D6.A3) was conjugated to horseradish peroxidase (HRP) using a conjugation kit (AB102890, Abcam Inc, Cambridge, Massachu-

setts 02139, USA) and then diluted 1 : 1 in glycerol and stored at −20°C. Sumatran rhino liver ferritin isolated as described above served as the assay standard. Ferritin concentration was determined using the Bradford assay with purified horse spleen ferritin (HF) of a known concentration (as reported by Sigma) replacing bovine serum albumin (BSA) as the standard. High- and low-quality control samples were made by diluting the standard with EIA buffer (PBS containing 5 µl/ml Tween 20 and 1% BSA) to the desired concentrations and storing aliquots frozen until use. Several checkerboard assays were run to determine the optimal concentrations of capture and indicator antibodies and to determine the linear range of the standard curve.

The final sandwich EIA protocol involved coating the wells with 100 µl of the capture antibody 5D10.B5 diluted to 1 µg/ml in coating buffer (1.325 mg/ml $Na_2HPO_4 \cdot 7H_2O$, 0.7 mg/ml $NaH_2PO_4 \cdot H_2O$, and 0.05 µl/ml Kathon). The plate was covered with an acetate plate sealer and incubated overnight at 4°C, then washed three times with EIA washing buffer (8.766 mg/ml NaCl and 0.5 µl/ml Tween 20 in ddH_2O) and blocked by adding 250 µl blocking buffer (PBS containing 1% BSA) per well and incubating at room temperature on a plate shaker under foil for at least 1 hr. The plate was washed three times and then samples, standards, and quality control samples diluted in EIA buffer were added to duplicate wells (100 µl/well). The standard curve consisted of seven serial dilutions ranging from 3,000 to 47 ng/ml. The high- and low-quality controls were 800 and 200 ng/ml of SRF standard, respectively. Sumatran rhino serum was typically diluted 1 : 3 or 1 : 5, but a few samples with very high or low ferritin concentrations were diluted 1 : 12 and 1 : 2, respectively, to ensure values fell within the linear portion of the curve. After all samples were added, the plate was sealed and placed on the plate shaker under foil for at least 1 hr before being washed three times. All wells received 100 µl of HRP-conjugated 1D6.A3 diluted to 400 ng/ml in EIA buffer. Plates were covered and shaken under foil for another hour and then washed three times a final time before adding 100 µl of substrate solution (1.6 mM hydrogen peroxide, 125 µl 0.4 mM azino-bis[3-ethylbenzthiazoline-6-sulfonic acid] in 0.05 M citrate buffer, pH 4.0) to each well. Plates were covered and placed under foil on the plate shaker until the color of the high standard developed to an optical density (OD) reading of ~1.0 (typically 1 hr) at which point the plate was read by a photospectrometer plate reader (Dynex MRX Revelation, Chantilly, Virginia 20151, USA) at a wavelength of 405 nm. A small subset of matched serum samples was also sent to Kansas State Veterinary Diagnostic Center (Kansas State University, Manhattan, Kansas 66506, USA) for ferritin analysis using the equine based assay previously validated for black rhinoceros serum ferritin.[46]

Animals and sample collection

A total of 13 Sumatran rhinos were included in this study: 3 males (SB#28, SB#42, and SB#44) and 3 females (SB#27, SB#29, and SB#43) maintained in US zoos, 5 females (SB#7, SB#13, SB#15, SB#19, and SB#23) maintained at the Sumatran Rhino Conservation Centre (SRCC) in Sungai Dusun, Malaysia, and 2 females (SB#51 and SB#57) from Sabah, Malaysia, the latter of which was a recently captured wild rhino. All blood samples had been collected for other purposes according to previously published methods for Sumatran rhinos in US zoos[43] and in Malaysia,[53] and serum recovered from red-top tubes was stored frozen until opportunistically utilized for this study.

Two of the female rhinos in the study (SB#29 and SB#43) died of liver failure due to hemochromatosis, which was diagnosed postmortem at the Cincinnati Zoo. One of the two (SB#29) was wild caught as a young rhino, lived in US zoos for 18.5 yr, and gave birth to three healthy calves. The other female was the daughter of SB#29. It was born at the Cincinnati Zoo and died before its 10th birthday. The third female rhino in the United States (SB#27) was wild caught as an older adult and exhibited reproductive pathology in the form of a large uterine mass.[43] It lived 16 yr in US zoos before dying in its mid-30s of cardiac compromise. All three male rhinos in the study were in US zoos at the time of blood collection. Two of the males (SB#42 and SB#44) were born at the Cincinnati Zoo and were transferred to the Sumatran Rhino Sanctuary on the island of Sumatra at 6.5 and 8.5 yr of age, respectively. They are both alive and healthy at the time of this writing at 16 and 10.5 yr of age, respectively. The third male (SB#28) was another wild-caught rhino estimated to be at least 10 yr old when it arrived in the United States. This rhino lived at the Cincinnati Zoo for 22 yr and died of thyroid cancer in its 30s. Liver tissue biopsies from the three Sumatran rhinos that died at the Cincinnati Zoo were sent to Michigan State University's Diagnostic Center for Population & Animal Health (Lansing, Michigan

48824, USA) for trace nutrient analysis, including iron concentration.

Four of the five female rhinos sampled at Sungai Dusun had significant reproductive pathology in the form of cysts and masses in their uteri. One female (SB#7) had been captured pregnant and produced a female calf (SB#15), but none of these females became pregnant while in captivity from ~1986 to 2003. All five rhinos were considered healthy at the time of sample collection (February 2003) but did die acutely of trypanosomiasis about 9 mo later.[50] Both rhinos sampled in Sabah, Malaysia, in May 2014 had reproductive pathologies in the form of uterine cysts (SB#51) or large uterine tumors (SB#57). One female (SB#51) was euthanized in June, 2017 due to aggressive melanoma. The second female (SB#57) is still alive, but experiences intermittent vaginal bleeding, at times significant, due to the large, invasive tumors (Z.Z. Zainuddin, pers. comm.).

Statistical analyses

To determine when ferritin concentrations were significantly elevated from an individual's "normal" baseline concentrations, an iterative process was employed. The data set for each of the five profiled Sumatran rhinos in US zoos was tested against its own mean value and any values falling outside of ±1.5 SD were removed. This process was repeated until no values remained outside the standard deviation.[4] With the baseline value for each rhino established, values significantly higher could be identified as those +1.5 SD of baseline. Data from matched samples analyzed by the equine assay and the rhino assay were compared using a Student's paired *t*-test and the qualitative relationship between the two data sets was assessed using correlation coefficients. Mean serum ferritin concentrations were compared among the six US zoo–housed Sumatran rhinos, from which multiple samples were analyzed by a one-way analysis of variance followed by two-sample *t*-tests assuming unequal variances and using a Bonferroni correction with $P < 0.01$ considered significant. To determine if ferritin concentration increased with age (or time in captivity), a correlation coefficient was calculated for 270 serum samples collected over time from US zoo–housed Sumatran rhinos. Samples collected after signs of clinical illness were exhibited in any of the rhinos that died during the sampling interval for this study were omitted from this analysis. Although initial ages for SB#28 and SB#27 were estimates because the rhinos were adults when captured, the data would still represent an appropriate yearly increase in age over time in captivity.

RESULTS

Serum samples

A total of 298 serum samples were analyzed in this study. Multiple samples (range, $n = 6$ to $n = 128$) were included from each of the six rhinos in US zoos collected over a period of 2–17 yr per rhino and spanning an 18-yr period (1997–2015), whereas single samples collected in 2003 were analyzed from the five rhinos maintained in the SRCC in Sungai Dusun, Malaysia. Additionally, a single sample from a rhino maintained in Sabah, Malaysia, and two samples collected from a wild-caught female in Sabah, Malaysia, in 2014, were analyzed.

Ferritin extraction and validation by gel electrophoresis and staining

Approximately 5 mg of protein was extracted from each gram of liver tissue. Gel electrophoresis and staining verified that the protein was purified ferritin. The protein banding pattern for SRF matched the banding pattern of the purified HF despite the fact that the three protein bands (242, 480, and 720 kDa) of HF consistently migrated faster through the gel (Fig. 1a). The absence of additional protein bands proved sample purity. Both the 242-kDa and the 480-kDa bands of ferritin stained positive with potassium ferricyanide (Prussian blue), confirming the association of iron with the protein.

EIA validation and specificity

Monoclonal antibody binding to rhino ferritin was validated via Western blot analysis. The capture antibody (5D10.B5) bound to all three rhino and horse ferritin bands (Fig. 2a). In contrast, the 1D6.A3 monoclonal antibody was highly specific, binding only to the primary 242-kDa band of the rhino ferritin (Fig. 2b) and not to any of the horse ferritin bands.

A pooled serum sample from four Sumatran rhinos was serially diluted (neat to 1 : 128) and exhibited parallelism to the standard curve with a correlation coefficient of 0.99. Interassay variation was 11.9% and intra-assay variation was 3.9%. Specificity of the assay was established further by testing serial dilutions of the purified equine ferritin. Even at a concentration of 2,650 ng/ml of HF, the OD readings remained at

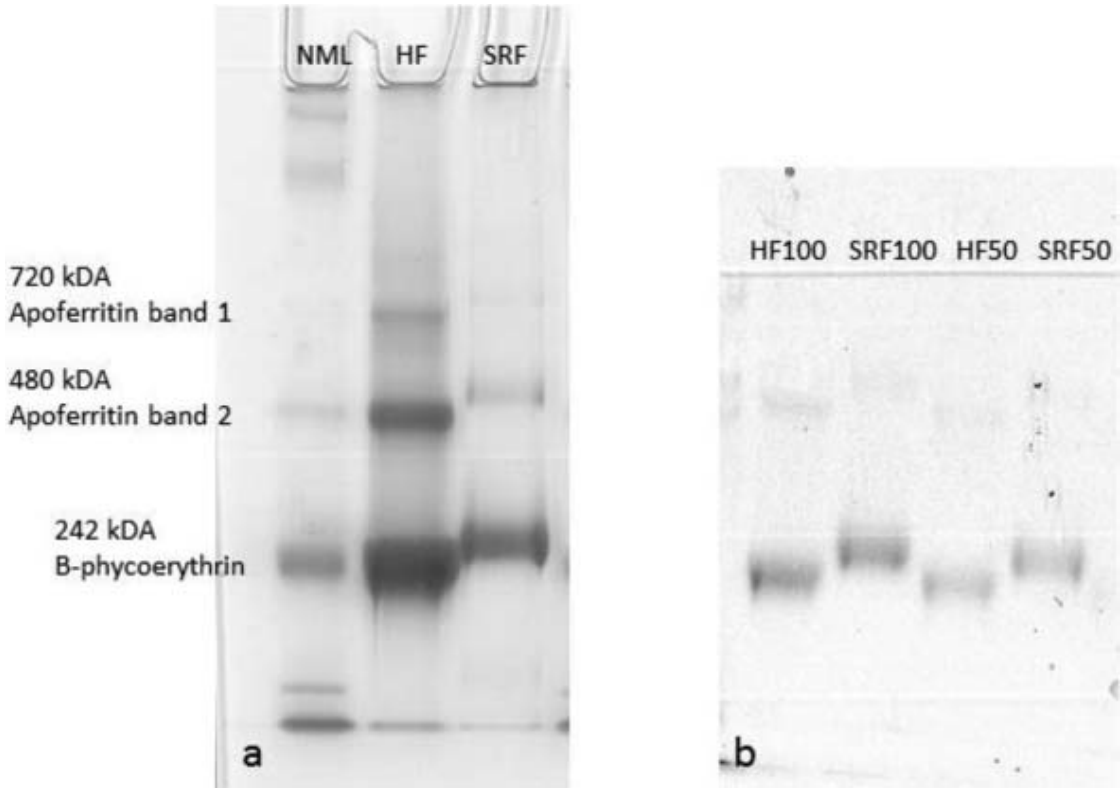

Figure 1. **a.** Coomassie blue staining of 3–8% tris-acetate gel containing (left to right) Native Mark Ladder (NML), purified horse spleen ferritin (HF) and Sumatran rhino ferritin extracted from liver tissue (SRF). **b.** Potassium ferricyanide staining of a similar gel containing 100 μg HF, 100 μg SRF, 50 μg HF, and 50 μg SRF.

background levels. Similarly, a llama and a cheetah serum sample inadvertently pulled from the serum bank and analyzed at a 1 : 3 dilution produced OD readings below the lowest value on the standard curve.

Serum ferritin profiles for Sumatran rhinos in US zoos

Mean concentrations of serum ferritin are reported as mean (±SD) and values above baseline are +1.5 SD above the individual's baseline. Across all US zoo samples, serum ferritin concentrations ranged from 348 to 7,071 ng/ml, and individual rhino mean concentrations ranged from 1,267 ± 227 to 2,604 ± 1,200 ng/ml. Neither the rhino with the lowest nor the one with the highest mean ferritin concentration died of hemochromatosis. Because of the dynamics of serum ferritin concentrations, longitudinal profiles were more insightful than lifetime means. Longitudinal profiles ranging from 3 to 15 yr were established for five of the six Sumatran rhinos in US zoos. Individual baseline values ranged from 1,046 to 1,984 ng/ml for four of the five rhinos. Data for the fifth rhino, SB#43, included many prepubertal samples, which resulted in a low overall baseline (551 ng/ml). If only samples collected during its adult life (≥4 yr) were included in the analysis, its baseline was much higher (3,559 ng/ml; Fig. 3c). All rhinos exhibited spikes in their serum ferritin concentrations above baseline values (Figs. 3, 4), and these spikes were not associated with clinical symptoms or onset of disease. The reproductive female exhibited a distinct pattern of baseline or near-baseline concentrations of ferritin during gestation and spikes above baseline after parturition and before the next conception (Fig. 4).

The most stable profile was exhibited by SB#27, an old, nonreproductive female that had been in the United States for 8–11 yr during the sampling period and was estimated to be 25–35 yr of age (Fig. 3a). Its ferritin concentrations ranged from 1,013 to 2,039 ng/ml with a mean of 1,267 ± 227 ng/ml (n = 25). This female died of cardiac compromise 5 yr after the last sample was

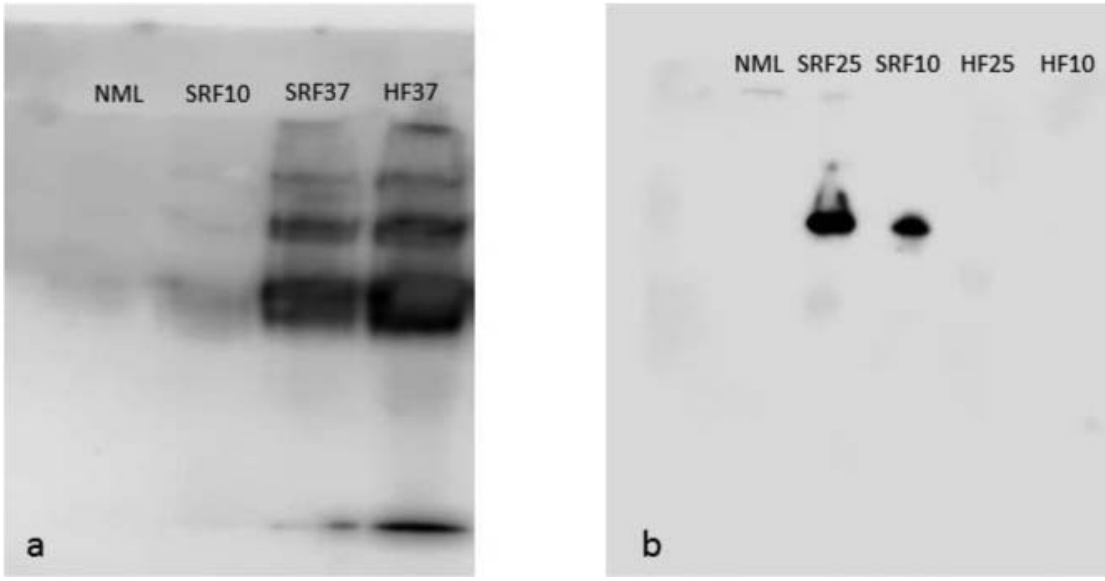

Figure 2. Western blot gels demonstrating the binding specificity of the two Sumatran rhino ferritin (SRF) monoclonal antibodies: (**a**) 5D10.B5 and (**b**) 1D6.A3. From left to right, columns contain (**a**) Native Mark ladder (NML), 10 and 37 μg SRF extracted from liver tissue, and 37 μg purified horse spleen ferritin (HF); (**b**) NML, 25 μg SRF, 10 μg SRF, 25 μg HF, and 10 μg HF. 1D6.A3 bound only the thickest protein band of the rhino ferritin.

collected for this study. Only mild, diffuse hemosiderosis was reported in the liver and spleen postmortem, with mild to moderate hemosiderosis found in the small intestine. A second relatively consistent ferritin profile was exhibited by SB#44, a young male rhino that was born at the Cincinnati Zoo (Fig. 3b) and spent time growing up at two other facilities. This male was 3 mo to 8 yr old during the sampling period and its mean ferritin concentration was 1,571 ± 524 ng/ml (range, 348–3,032 ng/ml; $n = 44$). Although there were some spikes above its baseline, the majority of this rhino's samples contained <2,000 ng/ml ferritin.

Two of the rhinos (SB#43 and SB#28) exhibited similarly dynamic profiles with progressively increasing serum ferritin concentrations over their lifetime (Fig. 3c, d, respectively). SB#43 was born and raised at the Cincinnati Zoo. This rhino was 2–9 yr old during the study and its mean ferritin concentration was 2,254 ± 1,487 ng/ml (range, 387–4,958 ng/ml; $n = 51$; Fig. 3c). This rhino did exhibit an increase in serum ferritin following the initiation of clinical symptoms of disease. Its highest serum ferritin concentration was recorded from a sample collected 2 days before it died of hemochromatosis, with severe hemosiderosis in the liver and moderate hemosiderosis in the spleen, kidney, and intestine reported postmortem. SB#28 was sampled for approximately 17 of its 22 yr at the Cincinnati Zoo, during which the rhino sired three calves. Its mean ferritin concentration was 2,603 ± 1,200 ng/ml (range, 950–5,982 ng/ml; $n = 36$; Fig. 3d). Its sample containing the highest ferritin concentration was collected 3 days before it was euthanized for medical reasons. Thyroid cancer was identified as the cause of illness postmortem, and only mild hemosiderosis was noted in the liver and spleen.

The most extensive profile was produced for SB#29, which was sampled during 12 yr of its life at the Cincinnati Zoo. This rhino was approximately 9–21 yr of age during the study period and gave birth to three calves during that time. This female's mean ferritin concentration was 1,614 ± 1,002 ng/ml (range, 751–7,071 ng/ml; $n = 128$; Fig. 4). The sample with the lowest ferritin was collected at the start of the third trimester of its third pregnancy, whereas the sample with the highest concentration was the last one analyzed 12 days prior to its death of hemochromatosis. Hemosiderosis was severe in the liver and moderate in the kidney, spleen, and intestine postmortem. This female's profile was the most dynamic, with serum ferritin elevated during periods of lactation and generally at baseline during pregnancy (Fig. 4). Similar to that for SB#43, this profile exhibited an increase in values after, but not prior to, onset of clinical symptoms of disease.

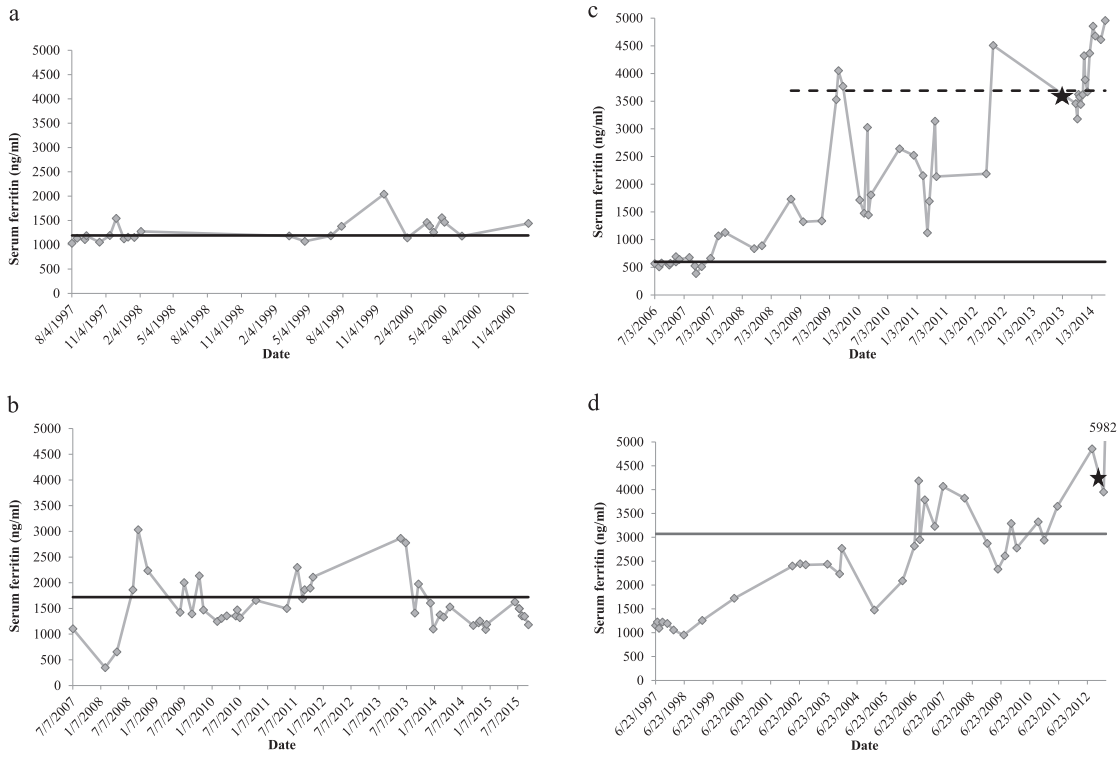

Figure 3. Longitudinal serum ferritin profiles for four Sumatran rhinos; solid lines represent the individual's baseline ferritin concentrations + 1.5 SD. **a**. SB#27, an old, nonreproductive, healthy female that died in 2005 of age-related cardiac compromise. **b.** SB#44, a young, healthy male that is still alive as of this writing. **c.** SB#43, a young female that died of hemochromatosis in March 2014 (star indicates when clinical symptoms were first observed, and dashed horizontal line represents individual's adult baseline ferritin concentrations + 1.5 SD). **d.** SB#28, a successful sire that was euthanized because of thyroid cancer in 2013 (star indicates when clinical symptoms of illness were first observed).

Only six samples collected over 4 mo were available for the sixth US rhino included in the study. This young male rhino, SB#42, was born at the Cincinnati Zoo and grew up at the Los Angeles Zoo. The rhino was 5.5 yr of age when sampled and its mean serum ferritin was 1,701 ± 275 ng/ml (range, 1,390–2,211 ng/ml). This male is alive in Sumatra at 16 yr of age as of this writing and has sired two calves.

Rhino ferritin assay versus horse ferritin assay

A total of 39 samples from four rhinos were analyzed via both the rhino-specific EIA developed herein and an EIA developed for horses and validated for black rhino ferritin.[46] Quantitatively, results from the two assays differed ($P < 0.01$) when analyzed in a paired Student's *t*-test. However, overall data sets were positively correlated ($R = 0.844$; $P < 0.01$). Because many of the samples ($n = 24$) in this comparison were from a single rhino, its data were analyzed further (Fig. 5). Quantitatively, data differed ($P < 0.01$) between assays but were positively correlated overall ($R = 0.86$; $P < 0.01$). However, data were skewed by an abundance of samples analyzed during its period of clinical symptoms. When samples collected prior to clinical illness were compared between assays, they differed quantitatively ($P < 0.01$; Student's paired *t*-test) and there was no longer a positive correlation between the two data sets ($R = 0.311$; $P = 0.36$; Fig. 5).

Liver tissue iron content

Liver iron concentrations measured postmortem in the three Sumatran rhinos that died at the Cincinnati Zoo ranged from 9,915 to 25,693 µg/g (Table 1). The rhinos with the lowest and highest liver tissue iron concentrations had the highest and lowest mean serum ferritin concentrations, respectively.

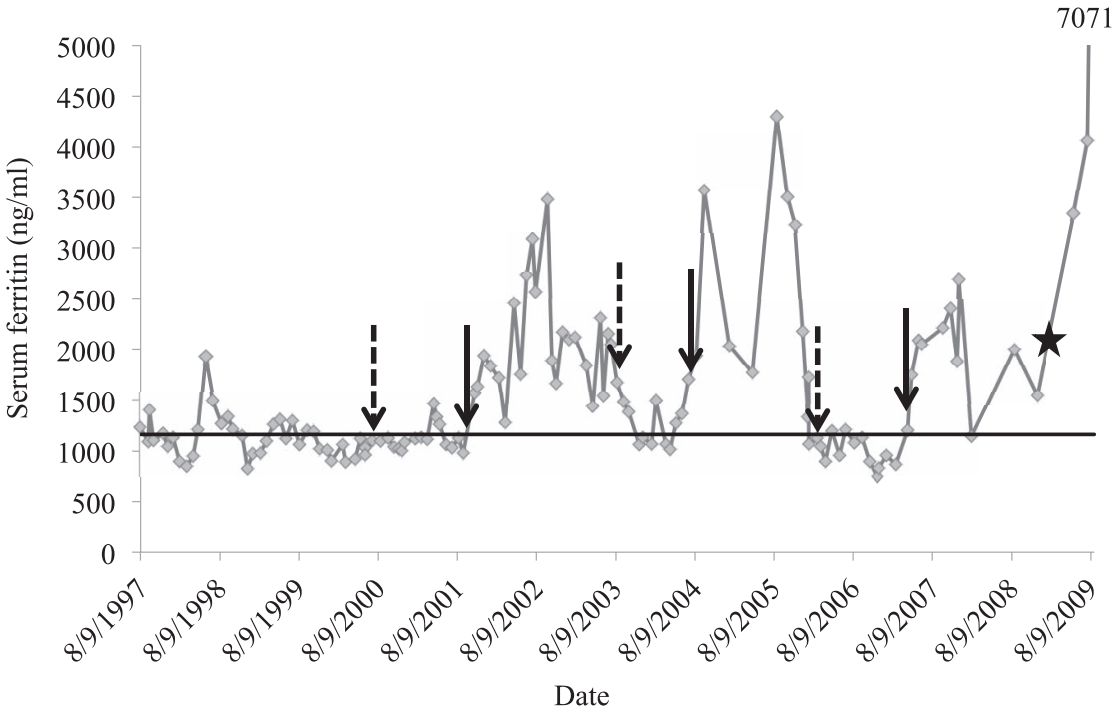

Figure 4. Serum ferritin profile for SB#29, a young, reproductive female Sumatran rhino that died of hemochromatosis in 2009. Dashed arrows indicate times of conception, solid arrows indicate dates of parturition, and the star indicates when clinical symptoms of illness were first noted. Solid line represents the individual's baseline value + 1.5 SD.

Serum ferritin in Malaysian rhinos

The mean ferritin concentration for Sumatran rhinos maintained in Malaysia was 4,904 ± 4,828 ng/ml (range, 987.5–14,900 ng/ml; Fig. 6), but one rhino had exceptionally high ferritin and was determined to be an outlier based on the Grubbs' test ($P < 0.05$). Even with that individual excluded, the mean value for the remaining Malaysian rhinos was high (3,238 ± 2,157 ng/ml; range, 987.5–5,632 ng/ml). However, no meaningful statistical comparisons could be made between the US and Malaysian rhino populations because of low sample numbers from the Malaysian rhinos. Interestingly, within Malaysia, the rhino populations appeared to have different ferritin concentrations, with those in Sabah averaging 1,025 ± 52.7 ng/ml compared to those in Sungai Dusun (6,456 ± 4,941 ng/ml; Fig. 6). Because SB#57 had been captured just weeks prior to sampling, its mean serum ferritin concentration of 987.5 ng/ml may be considered representative of wild, adult Sumatran rhinos. Although the Sungai Dusun rhinos all died in 2003,[50] information regarding the extent of hemosiderosis in the tissues of these rhinos is unavailable.

Serum ferritin concentrations and rhino age

Data from a total of 270 serum samples collected during the lives of the six Sumatran rhinos in US zoos were compared to the age, or estimated age, of the rhino at the time of sample collection (Fig. 7). The correlation coefficient was weak ($R = 0.22$), indicating that the two are not linked. Because time in captivity runs parallel to age of rhinos in this study, it is clear that time in captivity also is not associated with increased serum ferritin concentrations. Individual profiles in Fig. 3 further support these conclusions.

DISCUSSION

This retrospective study of the relationship between Sumatran rhino serum ferritin concentrations and IOD produced several unanticipated results. Of primary importance, the data do not support the use of serum ferritin for monitoring IOD progression or diagnosing hemochromatosis in the Sumatran rhinoceros. Mean serum ferritin concentrations were not higher in animals that died of hemochromatosis compared to those that did not develop the disease, nor were the profile

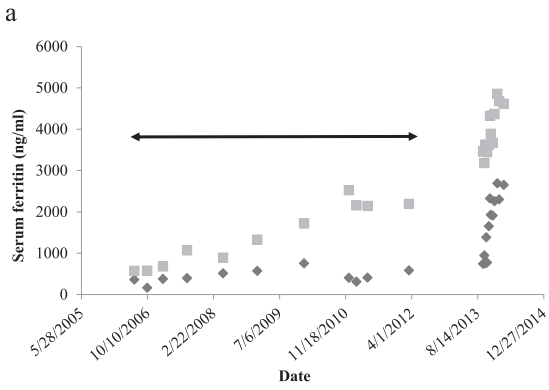

Figure 5. (**a**) Serum ferritin concentrations for Sumatran rhino SB#43 in matched samples analyzed by a horse ferritin assay (dark diamonds) versus the new rhino-phase assay (light squares), and (**b**) the same data in a log–log, x–y scatter plot with regression equation. Although the overall correlation coefficient ($R = 0.86$; $R^2 = 0.74$) suggested the values were positively correlated, an analysis of the samples collected prior to clinical signs of illness (denoted by black arrow in **a**) did not yield a meaningful correlation between the two data sets ($R = 0.31$; $R^2 = 0.09$).

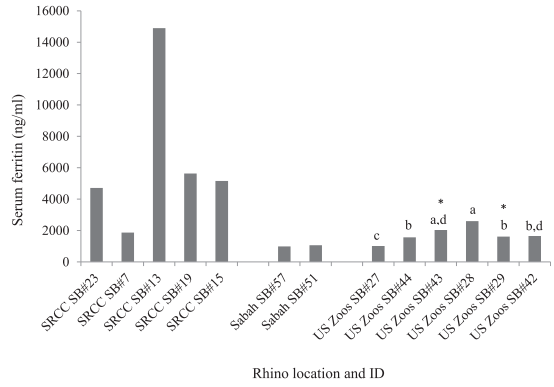

Figure 6. Mean serum ferritin concentrations measured in five Sumatran rhinos at the Sumatran Rhino Conservation Centre (SRCC) in Sungai Dusun, Malaysia ($n = 1$ sample per rhino), one wild-caught ($n = 2$ samples) and one captive ($n = 1$ sample) Sumatran rhino in Sabah, Malaysia, and six Sumatran rhinos maintained in US zoos (from left to right; $n = 25$, 44, 51, 36, 128, and 6 samples). Columns with asterisks denote rhinos that died of hemochromatosis. Columns with different superscripts differ ($P < 0.01$).

patterns consistent within the two groups. Contributing to the diagnostic challenge was the high degree of fluctuation in ferritin concentrations between sample collections, months, and years within individuals. All five rhinos for which profiles were generated exhibited spikes above their individual baselines during the monitoring period regardless of disease state, and ferritin concentrations in those that did develop the disease were not notably elevated when symptoms commenced. Additionally, liver iron load was not correlated with either mean serum ferritin concentrations or concentrations in samples taken just prior to death. Finally, with the exception of one individual, serum ferritin concentrations in rhinos maintained at Sungai Dusun, Malaysia, were high compared to those for the rhinos maintained in US zoos, and none of the Malaysian rhinos died of hemochromatosis.

Although elevated serum ferritin concentration is not a reliable biomarker of hemochromatosis in Sumatran rhinos, ferritin monitoring may not be without value. Consistently low values and less dynamic profiles could indicate the animal is not at high risk of developing the disease. Some studies in humans with hemochromatosis have also revealed a weak association between serum ferritin and body iron stores,[6] and in the horse, individuals can also deviate from expected values.[48] Similar to this study's findings, only false positives and not false negatives were reported.

Of particular interest was the similarity in profile patterns of SB#43 and SB#28, because SB#28 did not die of hemochromatosis and

Table 1. Serum ferritin, postmortem liver tissue iron content and, cause of death for three Sumatran rhinos.

Rhino ID (age at death [yr])	Overall mean serum ferritin (ng/ml)	Serum ferritin (No. days) prior to death	Postmortem liver tissue iron content (μg/g)	Cause of death
SB#28 (~32)	2,604	5,982 (3)	9,915	Thyroid cancer
SB#29 (21)	1,614	7,071 (12)	25,693	hemochromatosis
SB#43 (9)	2,254	4,958 (2)	19,646	Hemochromatosis

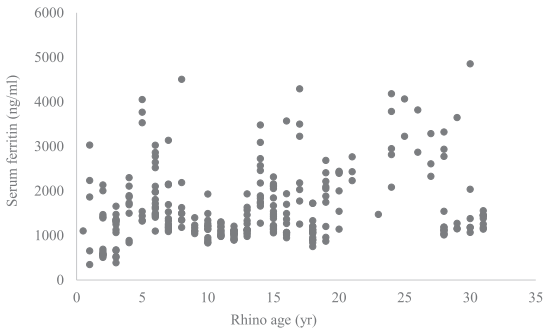

Figure 7. Sumatran rhino serum ferritin concentrations in samples collected from six individuals at different ages throughout their lives in US zoos. The relationship between serum ferritin concentrations and age is weak ($R = 0.22$).

exhibited only mild hemosiderosis postmortem. Ferritin increases in response to tumor necrosis factor independent of iron status[38] and is elevated in humans afflicted with several types of cancer,[1] but not thyroid cancer,[18] which was this rhino's cause of death. Furthermore, serum ferritin concentrations were elevated 7 yr prior to its death.

The Sungai Dusun rhinos died of trypanosomiasis,[50] and detailed postmortem assessments of organ hemosiderin content were not reported. Therefore, it is possible that the high serum ferritin was associated with significant organ iron stores, but none of the rhinos were sick with hemochromatosis. Alternatively, ferritin could have been elevated in response to growing tumors. Four of these five rhinos exhibited significant reproductive pathology including masses within their tracts. However, uterine fibroids typically cause bleeding, which lowers iron and ferritin concentrations,[11] and the US zoo rhino (SB#27) with a large uterine mass[43] exhibited the lowest and most consistent ferritin concentrations of those profiled.

The cyclical ferritin profile exhibited by SB#29 (Fig. 4) was another important finding suggesting that pregnancy impacts serum ferritin concentrations in Sumatran rhinos. Although pregnant women take supplemental iron to counter increased demand during gestation, rhinos do not receive similar supplements during pregnancy and must utilize body iron stores. The consistent decrease in ferritin during gestation and subsequent increase postpartum in this female suggest a beneficial role of pregnancy in reducing body iron in susceptible individuals. Although repeated pregnancies at relatively short 3-yr intervals did not prevent this female's eventual death from hemochromatosis at ~21 yr of age, they may have contributed to the rhino's longevity. Its daughter (SB#43) that never conceived succumbed to the disease at half the age. Interestingly, the only rhino at Sungai Dusun that was pregnant when captured had the lowest serum ferritin concentrations (<2,000 ng/ml). The importance of considering the confounding factor of pregnancy when measuring ferritin in serum samples from female rhinos has not previously been noted.

Significant profile fluctuations over time were also observed for nonpregnant rhinos, despite the fact that diets were consistent, no associated health issues were reported, and none of them were being treated for IOD via phlebotomies or chelation therapy, with the exception of SB#43 after clinical symptoms were observed. Given the apparent dynamics of ferritin concentrations in this species, long-term serial sampling is essential for monitoring individual status and impacts of dietary and environmental changes or IOD treatments on ferritin concentrations.

Despite the dynamics of the rhino ferritin profiles and the high single values of the Sungai Dusun rhinos, it is reasonable to conclude that healthy adult Sumatran rhino serum ferritin concentrations range between 1,000 and 2,000 ng/ml based on several pieces of evidence. First, samples from the wild-caught female in Sabah contained 858 and 1,117 ng/ml, which may reflect the lower normal range because this female was experiencing chronic vaginal bleeding from its fibroids. A sample from the second female in Sabah, which had only been in captivity for 2.25 yr and did not have uterine fibroids, contained 1,062 ng/ml ferritin. Additionally, the female in a US zoo that died in its 30s with mild hemosiderosis exhibited mean serum ferritin of 1,267 ng/ml. Finally, the mean ferritin concentrations for the two young males in US zoos were both below 2,000 ng/ml (1,701 and 1,571 ng/ml) and neither has developed the disease. Although these values appear high compared to those previously reported for several domestic mammal species, including piglets (mean 20.8 ng/ml),[47] cats (25–134 ng/ml),[2] horses (70–250 ng/ml),[48] and dogs (80–800 ng/ml),[3] quantitative values for serum ferritin can differ depending on the specificity of the antibodies and the epitopes to which they bind, as well as the ferritin standard used in the assay.[13,45] Two EIAs employing different antibodies to dog ferritin produced significantly different values for healthy dogs (80–800 and 261–1,889 ng/ml).[3,13] Because data generated in this study were

the product of a newly developed EIA that relies on SRF monoclonal antibodies and assay standard, the quantitative values obtained from the serum analysis should be accurate, and most do fall within the expected healthy, normal range for mammalian species (100 μg/dl, ranging from 55 to 185 μg/dl).[36]

It has been well established that antibodies employed in a horse ferritin EIA cross-react with black rhino ferritin, and after substituting black rhino ferritin as the standard in this equine EIA, it has been considered the gold standard for assessing black rhino serum ferritin.[39,46] Analyses of Sumatran rhino serum also indicated that the assay would cross-react with SRF, but some inconsistency both in assay results and in values associated with rhinos that did and did not develop hemochromatosis prompted this study. The monoclonal antibodies produced for this assay differed in their specificities; the capture antibody was less specific, but the signal antibody was highly specific for an epitope on the thickest band of the rhino ferritin and did not cross-react with serum ferritin from horses, llamas, or cheetahs. However, a preliminary test of serially diluted black rhino serum indicated high cross-reactivity to black rhino ferritin (T.L. Roth, unpubl. data), so the particular epitope to which the signal antibody binds may be conserved within the rhino taxon. When values were compared across the matched samples that were analyzed by both equine and rhino assays, it was not surprising that they differed quantitatively because of better detection by the rhino-specific assay antibodies. Trends in the results were fairly well correlated for some rhinos, but less so for others. For example, the subtle trend of increasing ferritin from 2007 through 2012 in SB#43 prior to symptoms of illness went undetected by the equine assay, despite a well-correlated increase in concentrations during illness. Furthermore, samples collected from SB#28 in 2010 and 2011 were 10 times higher when analyzed in the rhino assay versus the equine assay. Two hypotheses were tested in this study: the first, that a species-specific assay would provide more accurate results was proven, but the second, that with a rhino-specific assay, serum ferritin concentrations would reflect the progression of IOD to hemochromatosis in Sumatran rhinos, was refuted.

Given the rarity of this species, large sample sizes are never available for study. However, the long-term monitoring of several rhinos for which many details regarding diet, reproduction and health are known make this a robust data set to add to the growing literature on IOD in wildlife species. This study's findings differ from those reported in previous publications in several ways. First, data do not support previous conclusions that serum ferritin increases with time in captivity or with rhino age.[31,39,46] Although ferritin values for the young calves tended to be lower (~500 ng/ml), once they reached 3 yr of age, the values were at adult concentrations and often did not continue to increase. Furthermore, ferritin did not consistently increase with time in captivity. Despite being in captivity for 16 yr, SB#27 had the lowest mean serum ferritin of US zoo rhinos, which was similar to that of a wild-caught Malaysian rhino. Compared to previously published values for liver iron content in African black (4,636 ± 5,473 μg/g) or Sumatran rhinos (4,960 ± 6,279 μg/g) in US zoos,[19] values reported herein appeared high and did not necessarily increase with age, because the oldest rhino (by more than a decade) contained the lowest liver iron concentrations.

The high concentration of ferritin measured in the Sungai Dusun rhinos was perhaps the most surprising result. A previous report indicated that ferritin in Sumatran rhinos maintained in US zoos was higher than that in rhinos maintained in Southeast Asia.[12] Reduced browse diversity and increased iron content in the US diet were speculated to be responsible for this difference. However, this study's findings indicate that mean ferritin concentrations of the US zoo rhinos evaluated in this study with a rhino-specific assay were substantially lower than the values for four of the five rhinos at Sungai Dusun, Malaysia, and were more in line with the values for rhinos in Sabah, Malaysia. Despite the fact that in humans dietary nonheme iron, iron supplements, and iron-rich food are not correlated with ferritin concentrations,[10] nor is nonheme iron considered to be a significant contributor to hemochromatosis,[5] it is broadly believed that high dietary nonheme iron can contribute to IOD progression in wild herbivores.[16,39] However, detailed analyses of the diets for Sumatran rhinos at the Cincinnati Zoo[22] and those in Sabah, Malaysia[20] were published years ago and indicated that the iron content was potentially lower in the US zoo diet. These data, in conjunction with the fact that the rhinos profiled in this study had very similar diets throughout their lives yet very different longevity and morbidity outcomes, argue against dietary iron content as a key cause of hemochromatosis in Sumatran rhinos.

Although dietary differences between browsing rhinos maintained in captivity and those in the wild

have been the focus of much rhino IOD research,[15,21,28,29] because of the diversity in diets among species susceptible to hemochromatosis when in captivity (ie, fish-eating dolphins, fruit-eating tropical birds, and browsing rhinos), it seems unlikely that a specific dietary component (or lack thereof) is the underlying cause of disease. A more likely common denominator is an altered iron uptake regulatory mechanism, as previously suggested by Claus and Paglia[16] that evolved by necessity in many wildlife species surviving on inherently low-iron diets and subject to large and/or constant parasite loads. When these species come under human care, parasites often are eradicated, and traumatic events (ie, conspecific fights, predator–prey interactions) that would lead to occasional blood loss are greatly reduced or abolished. Furthermore, reproduction often is controlled and reduced, eliminating yet another pathway for iron usage and loss. Under these "idyllic" conditions of animal care, the risk of anemia dissipates, and if the species is efficient at absorbing iron from a low iron diet and/or has lost its ability to regulate iron absorption because of lack of necessity in recent evolutionary history, then it would be susceptible to hemochromatosis. Therefore, research at the molecular level on mechanisms of iron absorption and their regulatory systems like that pursued by Beutler et al[7] may be more likely to shed light on the etiology of this disease in wildlife. Meanwhile, further research is needed to identify a biomarker or signature of biomarkers for accurately assessing the progression of IOD, because serum ferritin concentration does not appear to be very informative in some susceptible species.

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