



Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Increased inflammation and decreased insulin sensitivity indicate metabolic disturbances in zoo-managed compared to free-ranging black rhinoceros (*Diceros bicornis*)

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ARTICLE INFO

Article history:

Received 11 November 2014

Revised 6 May 2015

Accepted 7 May 2015

Available online 14 May 2015

Keywords:

Black rhinoceros
Hemochromatosis
Glucose
Inflammation
Insulin
Iron overload

ABSTRACT

Black rhinoceros (rhinos) living in zoos express a host of unusual disease syndromes that are associated with increased morbidity and mortality, including hemolytic anemia, rhabdomyolysis, hepatopathy and ulcerative skin disease, hypophosphatemia and iron overload. We hypothesized that iron overload is a consequence and indicator of disturbances related to inflammation and insulin/glucose metabolism. The objectives of this study were to: (1) generate the first baseline information on biomarkers of inflammation (tumor necrosis factor alpha [TNF α], serum amyloid A [SAA]), insulin sensitivity (insulin, glucose and proxy calculations of insulin sensitivity), phosphate and iron stores (ferritin) using banked serum from free-ranging black rhinos; and (2) then compare serum biomarkers between zoo-managed ($n = 86$ individuals) and free-ranging ($n = 120$) animals. Enzyme immunoassays were validated for serum and then biomarker levels analyzed using mixed models while controlling for sex, age and year of sample collection. Concentrations of TNF α , SAA, insulin and insulin-to glucose ratio were higher ($P < 0.05$) in black rhinos managed in *ex situ* conditions compared to free-living counterparts. Findings indicate that the captive environment is contributing to increased inflammation and decreased insulin sensitivity in this endangered species.

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1. Introduction

The African black rhino (*Diceros bicornis*) is critically endangered, having declined by >90% from 100,000 individuals in the 1960s to ~5000 animals today, largely due to poaching (Emslie, 2013). The worldwide *ex situ* population of ~250 black rhinos (Emslie, 2012) also is under threat, chiefly due to a unique vulnerability to unusual disease syndromes. These maladies include hemolytic anemia (Miller and Boever, 1982; Paglia, 1993; Paglia et al., 1986), rhabdomyolysis (Murray et al., 2000), hepatopathy (Paglia and Tsu, 2012) and ulcerative skin disease (Munson et al., 1998), which contribute to morbidity and mortality (Carlstead

et al., 1999; Dennis, 2004; Dennis et al., 2007). Iron overload, or hemosiderosis, is one of the most commonly reported conditions, commonly diagnosed by measuring serum ferritin and iron, total iron binding capacity and transferrin saturation (Kock et al., 1992). At necropsy, abundant iron stores are found in many internal organs, including liver and spleen (Clauss and Paglia, 2012). We suspect that iron overload may be an indicator of underlying metabolic disorders.

Similar to humans, rodents and domestic animals, a sedentary lifestyle and increased fat repositories may create metabolic disturbances, including chronic inflammation, insulin resistance, iron overload, hypophosphatemia and hepatic disease (Haap et al., 2006; Rajala and Scherer, 2003; van Dijk et al., 2003; Xu et al., 2003; Yanoff et al., 2007). The horse, the closest domestic relative of the rhinoceros (Vaughan et al., 2000), commonly develops inflammation and insulin resistance with increased lipid deposits that result in a host of disease states (Field and Jeffcott, 1989; Johnson, 2002; Vick et al., 2006, 2007).

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Growing evidence suggests a correlative relationship between inflammation and insulin resistance (Rajala and Scherer, 2003; Ramos et al., 2003; Xu et al., 2003). Inflammatory cytokines, such as tumor necrosis factor alpha (TNF α), play a key role in regulating insulin/glucose homeostasis by inhibiting insulin signaling and glucose uptake, causing hyperglycemia and compensatory hyperinsulinemia (Hotamisligil et al., 1996; Lang et al., 1992). Through these mechanisms, cytokines are directly implicated in the development of insulin resistance. In humans, circulating acute phase protein and inflammatory cytokine concentrations are increased in overweight individuals and are even greater in obese patients with insulin resistance (Xu et al., 2003; Yang et al., 2006).

Interactions among inflammation, insulin/glucose metabolism and iron metabolism are complex (Fig. 1). Inflammation and insulin resistance adversely influence iron metabolism in humans. Iron overload has also been documented in the horse (Lavoie and Teuscher, 1993; Pearson et al., 1994; Smith et al., 1986), and increased serum ferritin has been associated with increased serum insulin and insulin resistance (Kellon, 2006; Nielsen et al., 2012). Inflammatory molecules, such as TNF α and acute phase proteins, divert iron from red blood cells by up-regulating ferritin expression and enhancing iron sequestration in tissue (Balla et al., 2007; Hirayama et al., 1993; Yanoff et al., 2007). Insulin also contributes to iron storage by stimulating cellular iron uptake and up-regulating ferritin synthesis (Davis et al., 1986; Yokomori et al., 1991). The relationship between iron and insulin/glucose metabolism also can be bi-directional. Iron interferes with insulin's inhibition of glucose production by the liver, thereby increasing serum glucose and augmenting hyperinsulinemia (Dandona et al., 1983; Niederau et al., 1984; Schafer et al., 1981). Iron stores also are positively correlated with insulin resistance in humans, even without significant iron overload (Fernandez-Real et al., 1998; Jehn et al., 2004; Jiang et al., 2004; Schafer et al., 1981; Tuomainen et al., 1997).

Inflammation and insulin resistance in humans also can contribute to hypophosphatemia (Gaasbeek and Meinders, 2005). Exogenous administration of cytokines causes marked decreases in serum phosphate levels (Barak et al., 1998), and insulin also stimulates cellular phosphate absorption, thereby decreasing serum phosphate availability. Like iron, hypophosphatemia can inhibit glucose uptake, exacerbating hyperglycemia and hyperinsulinemia (Haglin et al., 2001). Symptoms resulting from hypophosphatemia include hemolytic anemia, rhabdomyolysis, liver failure and neurological manifestations, including

encephalopathy (Gaasbeek and Meinders, 2005; Nanji and Anderson, 1985).

These disease conditions in humans are similar to those afflicting captive black rhinos, including rhabdomyolysis, liver disease and neurological symptoms (Dennis, 2004; Paglia et al., 2001). Hemolytic anemia is cited as a leading cause of death in captive black rhinos with a 75% mortality rate (Miller and Boever, 1982; Paglia et al., 1996). Hypophosphatemia also has been documented in multiple cases of black rhino illness (Murray et al., 2000; Paglia, 1993; Paglia et al., 1996). A survey of 88% of black rhinos held in North American zoological institutions from 1930 through 2001 revealed that 73% of captive-born animals died before reproducing, and 77% of these were <6 y of age (i.e., prepubertal; Dennis, 2004).

Our objective was to establish and compare values for inflammatory status and insulin sensitivity related to phosphate and iron stores in zoo-housed versus wild black rhinos. Determining reference intervals for these parameters for the first time from wild black rhinos was of particular importance. As the phenomena of inflammation and insulin resistance are complex (Malle and De Beer, 1996; van Deventer et al., 1990), we focused on multiple serum biomarkers. One priority was TNF α , which is well established to increase in circulation with inflammation (Hesse et al., 1988; Ramos et al., 2003; van Deventer et al., 1990) and play a role in insulin resistance, with concentrations elevated in overweight humans, rodents and horses (Lang et al., 1992; Ramos et al., 2003; Ruan et al., 2003; Vick et al., 2007). A second priority was serum amyloid A (SAA), an established biomarker of inflammation expressed by human adipocytes and correlated with body mass index (Malle and De Beer, 1996; O'Brien and Chait, 2006; Sasaki et al., 2003; Suganami et al., 2005). SAA is known to stimulate lipolysis, thereby contributing to insulin resistance (Malle and De Beer, 1996; O'Brien and Chait, 2006; Sasaki et al., 2003; Suganami et al., 2005) and is elevated in patients with iron overload (Kirk et al., 2001). We measured insulin and glucose as indicators of insulin sensitivity, while taking advantage of reference metrics in healthy, insulin resistant and equine metabolic syndrome horses (Keen, 2013; Nadeau et al., 2006; Suagee et al., 2013), another Perissodactyla species. Finally, we examined serum inorganic phosphate and serum ferritin, an established marker of iron stores in mammals, including the black rhino (Fernandez-Real et al., 1998; Smith et al., 1984; Tuomainen et al., 1997). We hypothesized that black rhinos maintained in zoological conditions exhibit increased concentrations of inflammatory markers and decreased insulin sensitivity compared to free-ranging counterparts.

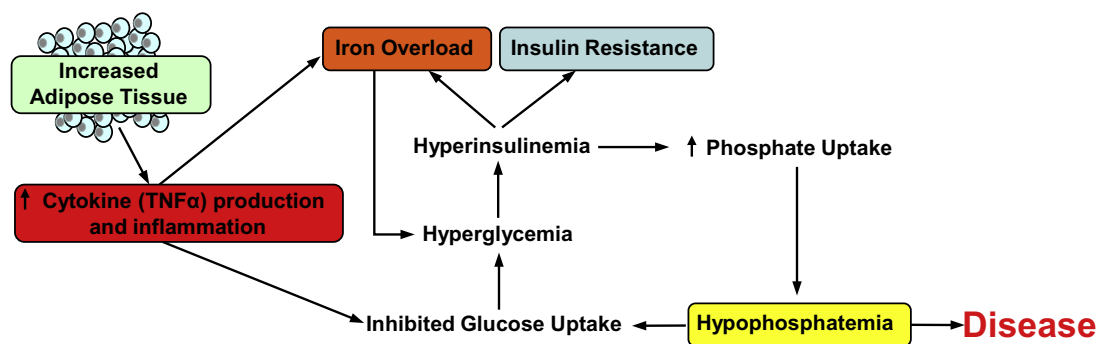


Fig. 1. Schematic of the hypothesized endocrine cascade leading to disease in zoo-managed black rhinos. Excessive adipose tissue creates a pro-inflammatory state and overproduction of cytokines that inhibit insulin-stimulated glucose uptake, thereby inducing hyperglycemia, consequent hyperinsulinemia and insulin resistance. Inflammation also diverts iron from red blood cell production into tissue storage. Hyperinsulinemia further contributes to iron overload by stimulating cellular iron uptake and up-regulating ferritin synthesis. Excess iron interferes with insulin's inhibition of glucose production by the liver, thereby increasing serum glucose and augmenting hyperinsulinemia. Insulin also stimulates cellular phosphate absorption, decreasing serum phosphate availability. The resulting hypophosphatemia exerts negative feedback by inhibiting glucose transport, exacerbating hyperglycemia and hyperinsulinemia. In humans, hypophosphatemia is correlated with hyperinsulinemia, insulin resistance and metabolic syndrome as well as diseases similar to those documented in the black rhino, including hemolytic anemia, rhabdomyolysis and encephalopathy (Gaasbeek and Meinders, 2005; Haap et al., 2006; Nanji and Anderson, 1985).

2. Materials and methods

2.1. Serum samples and biomarkers measured

This study was approved by all participating institutions and the animal care and use committee of the Cleveland Metroparks Zoo. Previously collected, frozen and stored black rhino serum was obtained from multiple sources. The fasting status at the time of serum collection was not known for the majority of animals; however, most were likely unfasted, as most zoo held animals were conditioned for blood draws, and most wild animals were darted with anesthetic under field conditions. Samples from free-ranging animals ($n = 77$ *Diceros bicornis minor*, $n = 12$ *D. b. bicornis*, $n = 31$ unknown) were provided by South African National Parks ($n = 48$) or the University of California-Davis (courtesy of Scott Larsen; $n = 72$). Samples were collected from rhinos classified as adult (>7 y), subadult (3–7 y) or juvenile (<3 y) mainly during translocation events. Serum was harvested after centrifugation of whole, clotted blood and stored frozen (generally at ≤ -20 °C) in individually-labeled cryovials.

For the comparative assessment, 12 North American zoos provided serum from 86 black rhinos of the *D. b. minor* ($n = 37$) or *Diceros bicornis michaeli* ($n = 49$) subspecies, all of known sex and age. Each was categorized into one of the three age groups, as described for free-ranging counterparts. For the captive population, serum was collected, frozen and stored as part of routine husbandry and medical management. In total, 726 samples were available, ranging from one to 41 samples per individual.

Serum provided by South African National Parks was analyzed at the Kruger National Park (Skukuza), whereas samples from the University of California and the North American zoos were evaluated at the Cleveland Metroparks Zoo. The same staff completed assays in both locations (Kruger National Park, Cleveland). Once received, all samples were thawed once to aliquot and evaluate for each metric. Internal controls were assayed to ensure intra- and inter-location consistency.

The stability of serum samples was examined to determine the influence of multiple freeze–thaw cycles. A subset of rhino samples ($n = 5$) was repeatedly thawed on the bench-top at room temperature for 20 min, assayed for each of the serum markers (Table 1) and then refrozen in a -20 °C manual defrost freezer for a total of six freeze–thaw cycles. For all biomarkers (except insulin and TNF α), serum values varied no more than 10% among freeze–thaw cycles. Both insulin and TNF α serum concentrations began to decrease by $>10\%$ after being frozen and then thawed four times. To our knowledge, serum samples used in our study were not thawed >3 times before analysis.

2.2. Hormone assays

Assays for commercial equine tumor necrosis factor alpha (TNF α ; Endogen, Rockford, IL), serum amyloid A (SAA, using equine standard equivalents; TriDelta Diagnostic Corp., Dublin, Ireland) and bovine insulin (Merckodia, Winston Salem, NC) were validated for black rhino serum. Recovery of serum spiked with high and low controls averaged 98.8, 91.1 and 96.3% for TNF α , SAA and insulin, respectively. Intra- and inter-assay coefficients of variation (CV) for TNF α averaged 7% and 11%, respectively; SAA, 2.8% and 13.4%, respectively; and for insulin, both 5.7%. Samples serially diluted in each assay's specified buffer exhibited parallelism with the standard curve (all P -values >0.05 ; TNF α $t = 0.985$; SAA $t = 0.49$; insulin $t = -0.092$). Serum was diluted at a range of: 1:3 to 1:4 for TNF α ; 1:10 to 1:400 for SAA; and neat to 1:30 for insulin. Linearity of sample dilutions (concentrations of hormone that varied no more than 80–120% between doubling dilutions) was achieved within the dilution range utilized for samples. Glucose and inorganic phosphate concentrations were determined from 40 μ l serum aliquots via a portable chemistry analyzer (Vet Test 8008 IDEXX Laboratories, Inc., Westbrook, ME). In brief, this involved pipetting 40 μ l serum from each animal into a polypropylene cuvette that was inserted into the analyzer concurrently with analyte-specific slides that allowed quantifying glucose or inorganic phosphate (Items 98-20369-US and 98-20374-US, respectively, IDEXX Laboratories, Inc.).

In humans, illness is associated with increases in inflammatory markers (Cohen et al., 2012; Cox, 2012; Watkins et al., 1995) and decreased insulin sensitivity (Carlson, 2003; Ligtenberg et al., 2001; Strommer et al., 1998). To demonstrate validity of the TNF α , SAA and insulin assays, serum samples from two rhinos with episodes of species-typical illness were analyzed. Within an individual, samples collected while unaffected by illness were compared to those collected while affected using a one-way T -test assuming unequal variance. Fig. 2A depicts mean concentrations of SAA and insulin as well as the I/G value from an adult male with extensive skin ulcerations and lethargy on an intermittent seasonal basis. Samples collected from 6 y while affected with the skin disease in winter ($n = 24$ samples) had higher ($P < 0.05$) SAA, insulin and I/G values compared to the unaffected season ($n = 25$ samples during summer). Fig. 2B depicts mean concentrations of TNF α , SAA, insulin and the I/G value for a 7 y old female before ($n = 10$ samples) and during ($n = 6$ samples) an episode of ulcerative skin disease and extended lethargy. The inflammatory markers were elevated ($P < 0.05$) during the disease period with a trend towards significance ($P = 0.07$) for decreased insulin sensitivity.

A ferritin enzyme immunoassay (EIA) was developed using general methods for the horse (Smith et al., 1984) and reagents from

Table 1
Median concentrations and 95% reference intervals for serum biomarkers in free-ranging black rhinos.

Biomarker (units)	Total number of animals	Number of animals (after outlier removal)	Median	Lower limit (90% CI) ^a	Upper limit (90% CI)
TNF α (pg/mL)	118	102	43.7	11.9 (9.9–15.2)	151.1 (111.4–183.5)
SAA (ng/mL)	120	104	5.6	0.8 (0.5–1.2)	82.7 (42.9–112.9)
Insulin (mU/L)	117	108	4.8	1.8 (1.6–2.1)	19.4 (15.6–22.7)
Glucose (mg/dL)	117	116	75.2	15.5 (11.4–20.6)	169.6 (161.1–190.7)
I/G (mU/mmol)	117	108	1.3	0.3 (0.27–0.38)	10.1 (7.8–12.1)
G/I (mg/dL/mU/L)	117	108	14.4	1.7 (1.2–2.4)	44.2 (39.1–48.8)
RISQI (mU/L) ^{-0.5}	117	108	0.5	0.1 (0.12–0.18)	0.7 (0.7–0.8)
QUICKI	117	104	0.4	0.3 (0.28–0.30)	0.66 (0.5–0.6)
Phosphate (mg/dL)	120	117	4.5	2.3 (2.1–2.5)	6.5 (6.2–6.9)
Ferritin (ng/mL)	120	112	293.8	53.9 (39.1–75.4)	893.1 (756.9–1007.1)

TNF α = tumor necrosis factor alpha; SAA = serum amyloid A; I/G = insulin-to-glucose ratio; G/I = glucose-to-insulin ratio; RISQI = reciprocal of the square root of insulin; QUICKI = quantitative insulin sensitivity check index.

^a CI = Confidence interval.

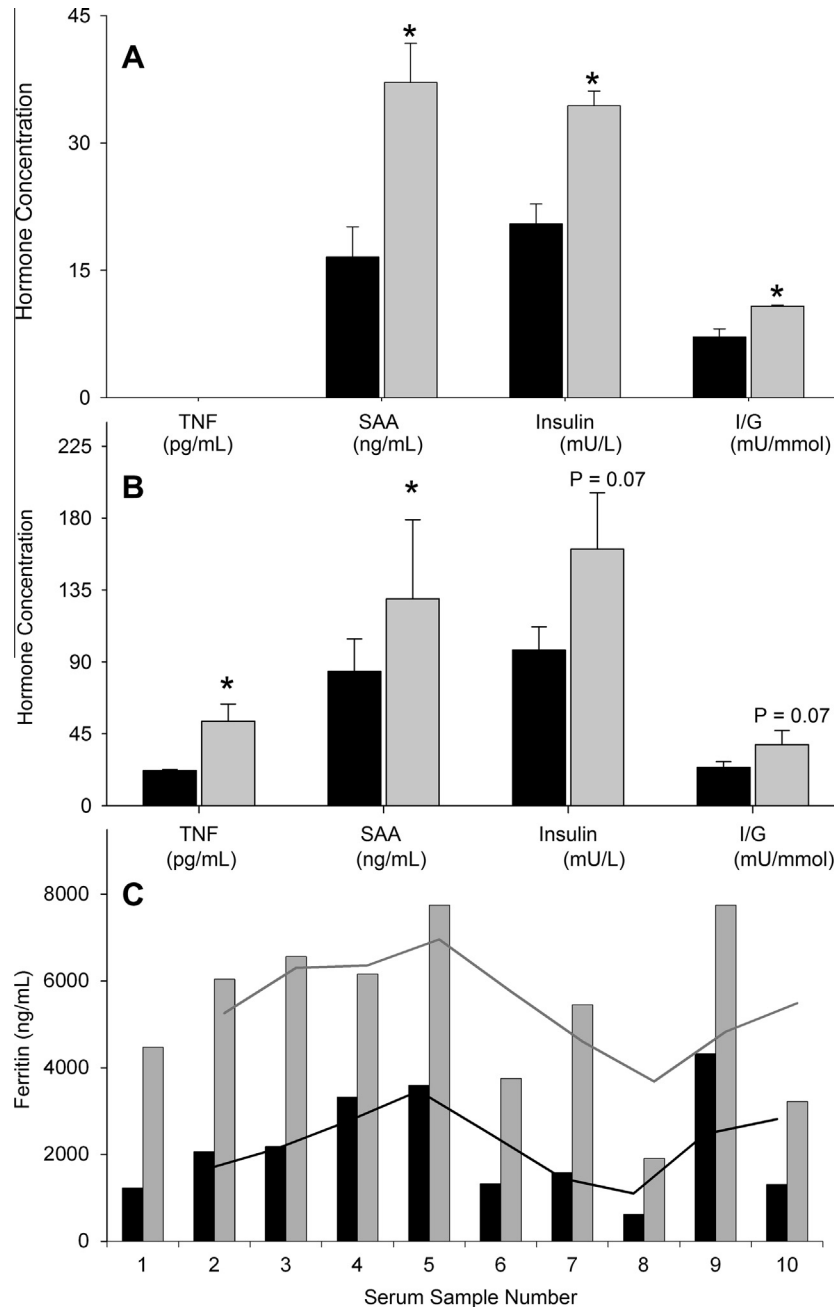


Fig. 2. Mean (\pm SE) serum concentrations of TNF α (Panel B only), SAA, insulin and I/G in: (A) an adult male black rhino while seasonally affected (grey bars, $n = 24$ samples) or unaffected (black bars, $n = 25$) by moderate to severe ulcerative skin disease and lethargy over 6 y and (B) a female while affected (grey bars, $n = 6$ samples) or unaffected (black bars, $n = 10$) by ulcerative skin disease and lethargy in a single year. Within an individual, an asterisk (*) indicates a difference ($P < 0.05$) between affected and unaffected samples. Panel C depicts serum ferritin values in 10 samples collected from 1998 to 2006 from different black rhinos. Black and gray bars, respectively, represent ferritin concentrations from the same serum samples assayed at Kansas State University using a black rhino ferritin standard versus in the authors' assay using an equine standard. The findings were correlated (Spearman $r = 0.95$, $P < 0.001$).

Sigma–Aldrich Corporation (St. Louis, MO) unless otherwise noted. After coating with antibody, plates were blocked for 1 h (37 °C) with a solution of 4% bovine serum albumin, 2% sucrose and 0.1% Tween 80 in Dulbecco's phosphate buffered saline (dPBS; pH 7.4, 0.2 micron-filtered) and were assayed immediately or dried, stored (–20 °C) and evaluated within 3 wk. Wash buffer consisted of 0.05% Tween 80 diluted in dPBS. The reagent diluent consisted of 4% BSA and 0.1% Tween in dPBS, and the peroxidase substrate and stop solutions were commercially prepared (KPL, Gaithersburg, MD). Equine anti-ferritin antibody was conjugated to horseradish peroxidase using a commercial kit (Surelink HRP conjugation kit; KPL). The same equine anti-ferritin AB antibody

(S-6146, Sigma Aldrich) was used as described by Smith et al. (1995) who validated a method and evaluated circulating ferritin in an earlier rhino study. For our project, ferritin was measured using a commercial, equine ferritin standard with results reported in equine equivalents. The assay was validated for black rhino serum with a minimum dilution of 1:2 (v/v). Serially diluted serum exhibited parallelism with the standard curve ($t = 0.762$, $P = 0.46$). Linearity was achieved in samples diluted from 1:2 to 1:1000. Samples were evaluated at 1:50 or 1:100 dilutions in reagent diluent with recovery averaging 101.0%. Intra- and inter-assay CV averaged 4.5% and 12.0%, respectively. For further validation, a subset of samples ($n = 10$) collected from 1998 to 2006 was split and

tested in our assay and at Kansas State University using the previously validated black rhino ferritin assay (Smith et al., 1995). While the units of each assay differed (equine standard vs. black rhino standard), ferritin concentrations from both assays were highly correlated (Spearman $r = 0.95$, $P < 0.001$; Fig. 2C).

We attempted to validate a serum leptin assay as a body condition marker, but were unsuccessful. Three commercial assays (Multi-Species Leptin Radioimmunoassay, EMD Millipore, Billerica, MA; Leptin EIA-2395, DRG International, Springfield, NJ; Mouse/Rat Leptin EIA, B-Bridge International, Inc., Cupertino, CA) and an ovine leptin EIA with antibody and procedures provided by H. Sauerwein (University of Bonn, Germany) were evaluated. While some assays passed parallelism, recovery and linearity validation, there was significant and inexplicable variation in cross-reactivity among individual rhinos from one assay to another. For example, serum from one zoo-managed female cross-reacted high on the standard curve of one assay, but serum from a more obese daughter produced a leptin concentration below detectable limits. When these same sera were re-analyzed using a different assay, findings were reversed. Because of this inconsistency, leptin values are not reported in this study.

2.3. Data analysis

To generate reference values from free-ranging rhino samples, we used the statistical analysis software of Horn and Pesce (2005). Briefly, we applied this non-parametric, robust estimation method following box-cox transformation and outlier removal. Additionally, indices of insulin secretion (insulin-to-glucose ratio, I/G) and insulin sensitivity (glucose-to-insulin ratio, G/I; quantitative insulin sensitivity check index, QUICKI; reciprocal of the square root of insulin, RISQI) validated for the human (Hancox and Landhuis, 2011; He et al., 1999; Katz et al., 2000; Kronborg et al., 2007; Sullivan et al., 2004), cat (Bjornvad et al., 2014) and horse (Frank, 2009; Kronfeld et al., 2005; Treiber et al., 2005) were calculated to allow cross-species comparisons. The insulin sensitivity index QUICKI was calculated as $1/[\log(\text{insulin}) + \log(\text{glucose})]$, and RISQI was determined as $\text{insulin}^{-0.5}$. To generate descriptive statistics for the population managed *ex situ*, all data points (including multiple observations per rhino) were used as a single data set. The mean, median, minimal (min) and maximal (max) values are presented to reflect entire data set variability. A median value also was generated from each individual for each biomarker. This enabled determination of the number of captive animals whose median value fell above or below relevant reference intervals in free-ranging counterparts or outside biomarker cut-off values reported in other mammals.

For further statistical analyses, serum biomarker concentrations and indices were box-cox transformed (Osborne, 2010) to approximate a normal distribution. Potential differences between serum biomarkers for zoo-maintained versus free-living rhinos were compared using a general linear mixed model (PROC MIXED; SAS version 9.3; SAS Institute, Cary, NC) while controlling for the potentially influential factors of sex, age category (adult, >7 y; sub-adult, 3–7 y; juvenile, <3 y of age) and interactions. Mixed models were run using a random intercept and slope (with date of the sample as the slope) with individual animal identification number and institution (zoo or wildlife preserve) included as random effects with the exceptions described below.

To evaluate insulin and glucose, a random intercept only was used for the random effect of institution while the random slope was omitted to achieve model convergence. Maximum likelihood approximation was used for model building, and non-significant factors were removed until the lowest -2 log likelihood, Akaike's information criterion (AIC) and Bayesian information criterion (BIC) were achieved. All factors were removed from the model at

$P > 0.10$ unless contributing to a lower -2 log likelihood estimate. Final models were run using restricted maximum likelihood estimation. Degrees of freedom were calculated using Satterthwaite's approximation. The effect of source (zoo-housed vs. free ranging) remained in the model at all times as this factor reflected our primary hypothesis. An unstructured variance-covariance structure was chosen based on AIC and BIC values that indicated best model fit. Cook's and Restricted Likelihood Distance tests were performed to identify potentially influential data points (outliers). Based on these results, three (of 843) data points were removed to evaluate I/G ratio, and 36 (of 846) points were removed to secure a final model with an acceptable residual structure when evaluating $\text{TNF}\alpha$. Analyses of residuals and random intercept effects (eblups) for normality were conducted with each final model. After box-cox transformation, dispersion and variance homogeneity requirements of parametric statistics were satisfactorily met for all serum biomarkers. When significant ($P < 0.05$) effects were determined, post hoc tests for multiple pair-wise comparisons were conducted using Tukey-Kramer adjustments of least squared means. Concentrations of biomarkers that differed between age and gender groups are reported as group medians in the text.

For purposes of reporting significant deviations from normal values, higher values of $\text{TNF}\alpha$, SAA, insulin and I/G indicated higher levels of inflammation and increased insulin resistance, whereas lower values of the proxies G/I, RISQI and QUICKI were considered indicative of insulin resistance. For comparative purposes, insulin resistance was defined based on studies in the horse and human. In the former, non-fasted concentrations of insulin >0.69 ng/mL (20 $\mu\text{U/L}$) reflect insulin resistance and equine metabolic syndrome (Frank, 2009; Keen, 2013). In the latter, the cut-off value for a fasting I/G ratio indicating impaired glucose tolerance is <0.25 mU/L/mg/dL (4.5 mU/mmol; Guerrero-Romero and Rodriguez-Moran, 2001). Established cut-off values in both fasted human samples and non-fasted horse samples for G/I, QUICKI and RISQI reflective of impaired insulin sensitivity are 4.5 (Divers, 2008; Kellon, 2007; Legro et al., 1998), 0.32 (Ascaso et al., 2003; Hřebíček et al., 2002; Treiber, 2006) and 0.32 (Treiber, 2006; Treiber et al., 2005, 2006), respectively.

3. Results

Reference intervals for serum biomarkers in free-ranging black rhinos are presented in Table 1. There were no differences ($P > 0.05$) between sample biomarker values generated from free-living individuals in the University of California-Davis collection compared to those from, and assayed in, South Africa. Within the wild population, there also were no differences ($P > 0.05$) between males and females or across age groups for any biomarker (data not shown).

Concentrations of both inflammatory markers, $\text{TNF}\alpha$ ($F_{(1,25)} = 4.4$, $P < 0.05$; Fig. 3A) and SAA ($F_{(1,220)} = 32.4$, $P < 0.001$; Fig. 3B), were higher in zoo-housed compared to the free-ranging animals. Insulin ($F_{(1,31)} = 59.9$, $P < 0.001$) and I/G ratio ($F_{(1,36)} = 45.0$, $P < 0.001$) also were higher ($P < 0.05$) in captive versus wild individuals (Fig. 3C and E), whereas glucose concentrations were similar ($P > 0.05$; Fig. 3D). The three indices of insulin sensitivity, G/I ($F_{(1,92)} = 55.3$, $P < 0.001$), RISQI ($F_{(1,206)} = 66.3$, $P < 0.001$) and QUICKI ($F_{(1,181)} = 58.9$, $P < 0.001$), all were lower in the zoo-held compared to free-living population (Fig. 3F–H, respectively). Finally, phosphate concentrations were lower ($F_{(1,60)} = 15.1$, $P < 0.001$; Fig. 3I) and ferritin higher ($F_{(1,85)} = 84.2$, $P < 0.001$; Fig. 3J) in the captive versus wild group.

There was no effect of gender ($P > 0.05$) on any of the biomarkers within the captive or wild population with the exception that $\text{TNF}\alpha$ was higher in zoo-housed females compared to zoo-housed

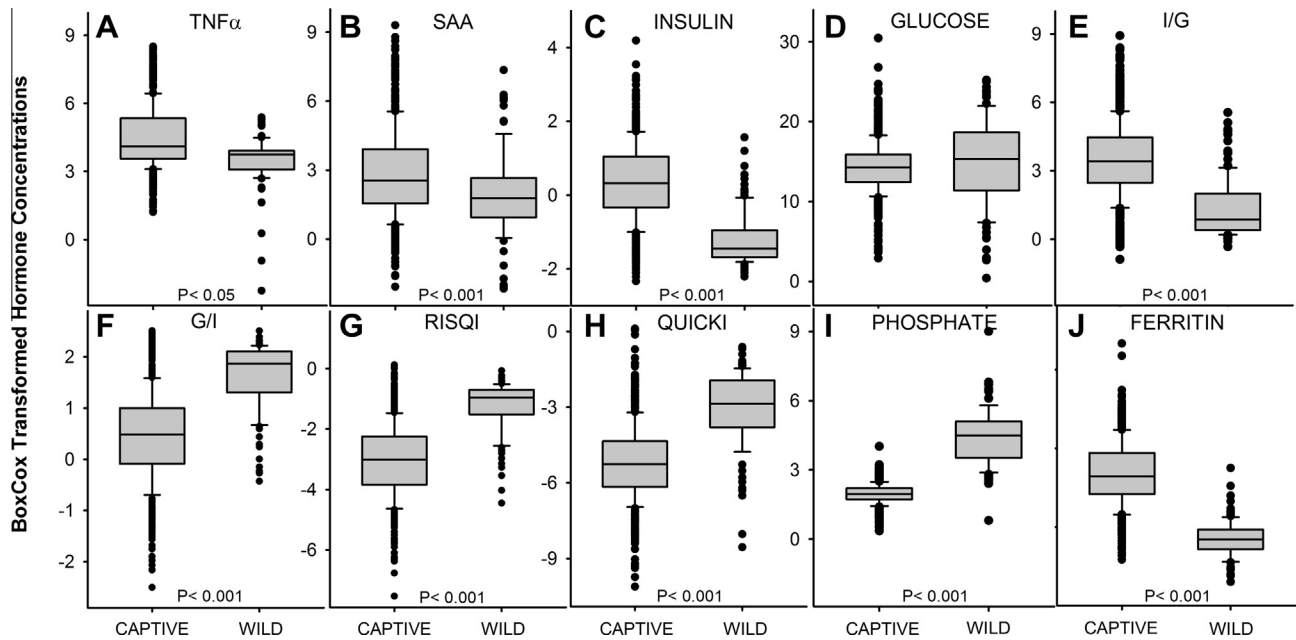


Fig. 3. Box and whiskers plots of box-cox transformed concentrations of: serum (A) tumor necrosis factor alpha (TNF α), (B) serum amyloid A (SAA), (C) insulin, (D) glucose, (E) insulin-to-glucose ratio (I/G), (F) glucose-to-insulin ratio (G/I), (G) the reciprocal of the square root of insulin (RISQI), (H) the quantitative insulin sensitivity check index (QUICKI), (I) phosphate and (J) ferritin in zoo-managed compared to free-ranging black rhinos. The bottom line of each box plot represents the 25th percentile in the distribution of scores, whereas the middle and top lines represent the 50th (or median) and 75th percentiles, respectively. The lower and top whisker represents the first and fourth quartiles of the data distribution, respectively, and the solid circles indicate outlying data points greater or less than 1.5 times the inter-quartile range. *P*-values at the bottom of each panel indicate significant differences between zoo-housed and wild populations for each biomarker.

males ($F_{(1,155)} = 13.9$, $P < 0.001$). Although not associated with serum TNF α and insulin or the insulin sensitivity proxies RISQI or QUICKI, age was related to other biomarkers. Post-hoc testing within the captive population revealed that juveniles had higher ($P < 0.05$) SAA concentrations (42.7 ng/mL) compared to subadults (9.3 ng/mL) or adults (10.3 ng/mL), a relationship that was lacking in free-ranging rhinos. The I/G ratio also differed by age ($F_{(2,346)} = 3.6$, $P = 0.03$) as adults and subadults had a higher ($P < 0.05$) values (10.2 and 12.1, respectively) than juveniles (5.2). Concurrently, the G/I ratio also was lower in adults (1.8) compared to juveniles (3.5) in all black rhinos regardless of being in captivity or the wild ($F_{(2,589)} = 5.8$, $P = 0.04$). Juvenile rhinos living in zoos also had higher phosphate concentrations (6 mg/dL) compared to subadults and adults (4.9 and 4.5, respectively), $T_{(234)} = 6.7$, $P < 0.001$, an observation not evident in wild individuals. There also was a source by age category interaction ($P < 0.001$) for ferritin, as median circulating concentrations increased from juvenile (1251 ng/mL) to subadult (1853 ng/mL) to adult (2895 ng/mL) rhinos in captive, but not wild black rhinos.

Percentage of zoo-managed rhinos with median values outside reference intervals for each of the serum biomarkers are shown in Table 2. Individual median concentrations of each serum biomarker were above free-ranging reference values for 21–39% of the 86 captive rhinos for TNF α , SAA, insulin and I/G ratio. Sixty-one percent (53 of 86 rhinos) in the *ex situ* collection had median insulin concentrations >0.69 ng/mL (reference value based on the domestic horse; see methods) compared to only 11% of free-ranging counterparts. Likewise, median I/G ratio values exceeded the cut-off metric for insulin resistance (>0.25 mU/L/mg/dL; 4.5 mU/mmol indicative of resistance; human reference, see methods) in 63% (54 of 86) captive rhinos compared to only 22% of the wild cohort. Similarly, individual median values for insulin sensitivity measures of G/I, RISQI and QUICKI fell below free-ranging reference intervals in 20–32% of the rhinos living in zoos (lower values reflecting insulin resistance). The median values

of these three indices also were below established cut-off values indicative of insulin resistance and metabolic syndrome in the human and horse (<4.5 , 0.32 and 0.32, respectively) in 55–74% of captive versus 10–23% of wild rhinos. Median phosphate concentrations for captive rhinos fell within reference intervals for most individuals of both populations. However, only 8% (seven of 86) of rhinos in zoos had median ferritin concentrations within reference intervals of the wild population. Of those seven animals, five were prepubertal (<4 y; four females, one male).

4. Discussion

This study was conducted because it is challenging to manage a healthy population of black rhinos *ex situ*. When exposed to traditional zoo environments, this species experiences serious medical conditions that result in high morbidity and mortality. There is irony here as some ‘unknowns’ compromise our ability to keep black rhinos healthy and reproducing in captivity while the wild population is simultaneously being decimated by human poaching. Thus, there is urgency in understanding potential origins of issues related to iron overload, rhabdomyolysis, liver and neurological diseases in the *ex situ* cohort so as to develop a sustainable population as ‘insurance’ against the potential of losing this species in nature. Our strategy was two-pronged with the first step being to validate biomarkers from growing evidence that certain indicators accurately reflect inflammatory status and insulin/glucose metabolism (Fernandez-Real et al., 2002; Hotamisligil, 1999, 2003; Ramos et al., 2003; Sasaki et al., 2003; Suagee et al., 2013; Sugaami et al., 2005). The second priority was to secure substantial numbers of serum samples from black rhinos of both genders and diverse ages representing both zoo-managed and free-living populations. Samples were obtained from more than 200 black rhinos living in zoos or the wild. After validating all but one of our targeted factors (leptin), data assessment supported the assertion that the captive environment was associated with a pro-inflammatory

Table 2
Descriptive values for serum biomarkers in samples from 86 zoo-managed black rhinos^a.

Biomarker (units)	Mean	Median	Min	Max	Percentage of rhino population outside reference intervals (No. out of total) ^b
					<i>Percent above interval^c</i>
TNF α (pg/mL)	282.0	51.5	0.1	6362.4	21% (18 of 86)
SAA (ng/mL)	192.2	12.4	0.1	10384.0	36% (31 of 86)
Insulin (mU/L)	55.2	39.3	0.8	506.1	39% (34 of 86)
Glucose (mg/dL)	70.37	65.5	6	575	1% (1 of 86)
I/G (mU/mmol)	17.3	11.2	0.2	149.1	33% (28 of 86)
					<i>Percent below interval^d</i>
G/I (mg/dL/mU/L)	4.5	1.6	0.1	77.3	32% (27 of 86)
RISQI (mU/L) ^{-0.5}	0.20	0.16	0.04	1.13	20% (20 of 86)
QUICKI	0.31	0.29	0.20	1.12	30% (26 of 86)
Phosphate (mg/dL)	5.05	4.9	1.4	16.1	2% (2 of 86)
Ferritin (ng/mL)	3643.6	2669.7	101.3	28485.6	92% (79 of 86)

TNF α = tumor necrosis factor alpha; SAA = serum amyloid A; I/G = insulin-to-glucose ratio; G/I = glucose-to-insulin ratio; RISQI = reciprocal of the square root of insulin; QUICKI = quantitative insulin sensitivity check index.

^a Descriptive statistics were calculated using the entire set of captive samples (786 samples from 86 rhinos).

^b Number of zoo-held rhinos with median concentrations above or below reference interval (based on the total number of captive animals in the study).

^c Percentage of zoo-housed rhinos with median concentrations above the upper limit of reference intervals for TNF α , SAA, insulin, glucose, I/G and ferritin.

^d Percentage of zoo-housed rhinos with median concentrations below the lower limit of reference intervals for G/I, RISQI, QUICKI and phosphate.

state and decreased insulin sensitivity in the black rhino. When combined with observed increases in circulating ferritin and decreased serum phosphate, findings indicated significant metabolic disturbance in this *ex situ* population. This contention was supported by multiple lines of evidence.

First, we observed group differences in established inflammatory mediators that have been described in humans as key links in developing metabolic syndromes and associated diseases (Wang and Nakayama, 2010). Although a cause-effect could not be proven from the present study, the 18% differential elevation in median serum TNF α in the zoo versus free-ranging rhino population was consistent with the discrepancy reported for people who were obese, insulin resistant or experiencing metabolic syndrome versus healthy individuals (Hivert et al., 2008). Modest but significant increases in serum TNF α also have been reported for obese women with impaired glucose tolerance compared to normal weight, healthy controls (2.88 vs. 1.65 pg/mL, respectively; Tsigos et al., 1999). One potential contributor to the difference in TNF α between captive and wild rhino populations that deserves future evaluation is diet, especially given the differences between standard zoo provisions of hay and commercial grain versus the browsing of indigenous plants in nature (Buk and Knight, 2010; Helary et al., 2012; Malan et al., 2012). As horses fed a commercial, high starch and carbohydrate diet also have elevated circulating TNF α compared to those receiving a fiber/fat-rich concentrate (Suagee et al., 2011), this sensitivity may be common to species in the order Perissodactyla and should be further evaluated.

Another important biomarker is SAA that is produced by adipocytes and well recognized to reflect obesity and body mass index (BMI) in the human (Danesh et al., 1999; Gómez-Ambrosi et al., 2008; Zhao et al., 2010). For example, serum SAA content in one study was 1.5-fold higher in obese versus healthy weight siblings (Yang et al., 2006). We observed an age effect on SAA concentrations, but only for animals living in zoos. In the domestic horse, circulating SAA is correlated to both insulin and body condition, with concentrations threefold less in individuals with ideal compared to overweight or obese body condition (Suagee et al., 2013). Although increased SAA indicates inflammation similar to TNF α , consistent elevations in this marker also may reflect excessive weight, which could be occurring in zoo-managed black rhinos. While we do not yet have direct evidence, diets fed to young black rhinos in zoos could have increased both adiposity and peripheral SAA values compared to older counterparts. Besides indicating inflammatory

status, SAA also has been associated with iron overload in other species. For example, Kirk et al. (2001) reported a threefold increase in mRNA expression of SAA in mice with dietary iron overload compared to controls. In that study, the diet was enhanced in iron, but not fat content, and the mice were not obese, suggesting a link between iron overload and inflammatory mediators independent of body condition. Therefore, it is reasonable to speculate that the increased SAA measured in zoo-maintained rhinos may have contributed directly to iron overload.

The consistently observed increases in circulating TNF α and SAA indicate that at least a pro-inflammatory state exists in zoo-managed black rhinos. Such conditions can drive profound endocrine disruptions in other species, including disturbed insulin-glucose metabolism. Therefore, we assessed non-fasting insulin and glucose as measures of insulin sensitivity, as used in population-based studies of humans (Hancox and Landhuis, 2011) and for routine diagnosis of insulin resistance and metabolic syndrome in the horse (Divers, 2008; Treiber et al., 2005). The non-fasting insulin reference interval for free-ranging black rhinos of 1.8–19.4 μ U/mL was similar to the 2–15 μ U/mL reported for the horse (Nadeau et al., 2006; Pagan et al., 2009). A remarkable 61% of black rhinos in zoos had median insulin concentrations above the level indicative of obesity, insulin resistance and metabolic syndrome in the horse (20 μ U/mL; Frank, 2009; Keen, 2013; Suagee et al., 2013). By contrast, median glucose concentrations did not differ between rhino populations, and values fell within the interval reported for the healthy domestic horse (Nadeau et al., 2006) and a few ($n=6$) zoo-maintained white rhinos (*Ceratotherium simum*; Berkeley et al., 2011). Only 15% of both wild and captive black rhinos had glucose concentrations exceeding the upper limit of the reference interval for the horse (Keen, 2013), indicating that hyperglycemia is not prevalent in either population. Similarly, hyperglycemia rarely occurs in horses with insulin resistance or equine metabolic syndrome (Keen, 2013).

The insulin-to-glucose ratio (I/G) for the black rhino was informative. More than half of zoo-managed animals had I/G values exceeding the metric identified in humans as the cut-off (0.25 mU/L/mg/dL; 4.5 mU/mmol) for impaired glucose tolerance (Guerrero-Romero and Rodriguez-Moran, 2001) and predisposition to hypertension and diabetes (He et al., 1999; Perry et al., 1999). More importantly, there was a 10-fold increase in median I/G ratios in captive compared to wild populations, strongly indicating impaired glucose tolerance in zoo animals. The age dependent

increase in I/G ratios observed in zoo-housed adults and subadults compared to juveniles supported the association of the captive environment over time to insulin resistance and progressive metabolic disturbance.

The G/I ratio and the QUICKI and RISQI indices are well-established indicators of insulin sensitivity. For example, a G/I ratio <4.5 in the human is suggestive of insulin resistance and metabolic syndrome (Legro et al., 1998), whereas a value <10 is considered abnormal in the horse with those <4.5 considered to be 'substantially' insulin resistant and at high risk for equine metabolic syndrome (Divers, 2008; Kellon, 2007). While 63% of captive and 20% of the free-ranging individuals produced median G/I values below the human/horse cut-off points, the more pronounced finding was the ninefold decrease between those animals living in zoos versus nature. The QUICKI assessments also supported evidence of insulin resistance in the captive population, with more than half of zoo-managed rhinos having median QUICKI values < the 0.32 cut-off for the human (Ascaso et al., 2003; Hřebíček et al., 2002), horse (Treiber, 2006; Treiber et al., 2005) and domestic cat (Bjornvad et al., 2014). Finding that 30% of the zoo-housed rhinos produced median values below reference values for wild counterparts also indicated a skew in the captive population. The RISQI proxy created specifically for the horse also has a cut-off score of 0.32 with lower values indicative of metabolic syndrome (The Liphook Equine Hospital, 2010; Treiber et al., 2006). Seventy-four percent of zoo-housed individuals fell below this value. However, so did 23% of free-ranging individuals, suggesting that the QUICKI assessment was more informative and consistent than RISQI for determining insulin sensitivity in this species.

Although circulating phosphate differed in the zoo-held versus free-ranging animals (4.5 vs. 4.9 mg/dL, respectively), the difference in median concentrations was only 0.4 mg/dL, and both populations were well within reference intervals. Hypophosphatemia is observed in human patients, with clinical signs only evident when serum phosphate is <1 mg/dL (Gaasbeek and Meinders, 2005). However, decreases in serum phosphate, even within normal intervals, correlate to reduced insulin sensitivity (Haap et al., 2006). When hyperinsulinemia is corrected, serum phosphate levels return to normal (Gaasbeek and Meinders, 2005). Our observations with rhinos may have reflected a slow trend towards hypophosphatemia over time, and it would be interesting to know if phosphate values would rebound in the face of greater insulin sensitivity. Here too we observed an age impact, but only in the captive cohort, with higher circulating phosphate in juveniles than adults. An analogous pattern of increased phosphate concentrations has been measured in children compared to human adults (Bansal, 1990).

Finding significantly higher serum ferritin concentrations in 95% of zoo-maintained black rhinos was consistent with earlier, smaller scale studies of this same species (Kock et al., 1992; Smith et al., 1995). Our results underscored the prevalence of this condition in zoo-managed programs >20 y after these original reports. The serum biomarker concentrations measured in black rhinos also reflected metabolic perturbations that were similar to what has been reported for the human (Guerrero-Romero and Rodriguez-Moran, 2001; Tsigos et al., 1999; Yang et al., 2006) and horse (Suagee et al., 2013; Treiber et al., 2005). These metabolic disturbances may exacerbate iron overload; for example, as health conditions deteriorate in people with metabolic syndromes, circulating ferritin increases linearly (Bozzini et al., 2005). Although metabolic alterations may amplify tissue uptake and iron storage in the black rhino, there appears to be a dietary connection. For instance, it is well established that diets fed in captivity result in more iron intake than ingested in free-living counterparts (Buk and Knight, 2010; Helary et al., 2012). There also is the possibility of a genetic influence, specifically a mutation in the hepcidin gene that may accentuate iron accumulation in tissue of this browsing

species (Beutler et al., 2001). Although a contributor to developing hereditary hemochromatosis in the human (Nemeth and Ganz, 2009), hepcidin gene alteration has not been apparent in the Sumatran rhino (*Dicerorhinus sumatrensis*), another browsing species that develops iron overload in zoos (Beutler et al., 2001).

For the black rhino, one theory is that this species evolved on a low iron diet and has no mechanism for preventing gastrointestinal absorption of excessive dietary iron (Beutler et al., 2001). Zoo-based diets may contribute not only to iron overload, but also inflammation and insulin resistance. In the wild, black rhinos feed mainly on woody shrubs, forbs and succulent plants (Dierenfeld et al., 1995; Hall-Martin et al., 1982) while zoo diets consist primarily of grass or lucerne hay and milled concentrate supplemented with fruits and vegetables (Clauss et al., 2006). These zoo diets represent a decrease in fiber and higher nutrient availability compared to the wild. Excess nutrient intake and low fiber diets increase inflammation (Suagee et al., 2011) and decrease insulin sensitivity in the horse (Pratt et al., 2006). While our study was retrospective and not designed to address diet, the relationship among diet, inflammation and insulin sensitivity warrants priority investigation in the near future.

This may be the first comparison of inflammatory markers and insulin/glucose status between any species managed in captivity versus the wild. It has illustrated the value of such a comparative analysis for identifying potential husbandry flaws that are preventing an endangered species from thriving under human care. For the black rhino, there were profound differences in all circulating, inflammatory and insulin/glucose biomarkers pointing to significant metabolic disturbance for animals managed in traditional zoological environments. That these observations were valid was supported by analogous findings in other species (especially the human and horse) that experience similar syndromes and disease. One other near-term priority will involve screening the medical cases of black rhinos kept in zoos and evaluated here, especially those individuals that were examined longitudinally with serial blood sampling. There is enormous untapped information to be learned from those cases now that we have established an extensive database of inflammatory and insulin/glucose marker values as well as circulating ferritin and phosphate concentrations. We suspect that some of these individuals will provide best clues for next investigative steps. Regardless, the other high priority target for more thorough examination is species body condition, with increased adiposity being a primary contributor to observed metabolic disruptions in other species. The target here should be to examine the role of diet and/or activity levels of black rhinos living in zoos. The former is much more practical to address than the latter. Multiple studies in horses have demonstrated that concentrates higher in fiber and fat and lower in sugar and starch have benefits to insulin sensitivity and health (Hoffman et al., 2003; Kronfeld et al., 2006). A similar study, especially examining the essential components associated with browse feeding, warrants immediate attention for best practices and health for black rhinos managed *ex situ*.

Acknowledgments

The authors thank zoological institutions contributing samples for analysis: Disney's Animal Kingdom, Denver Zoo, St. Louis Zoo, Columbus Zoo, Cleveland Metroparks Zoo, White Oak Conservation Center, Fossil Rim Wildlife Center, Milwaukee County Zoo, Miami Metro Zoo, Sedgwick County Zoo, Fort Worth Zoo, Great Plains Zoo and Brookfield Zoo. We also thank South African National Parks for access to serum, housing and laboratory space and Scott Larsen for generous access to serum samples from the University of California, Davis's free-ranging black rhino cryo-repository. We also thank Chris Peterson and Lindsey Long for assistance with sample analysis and Karen Grace-Martin of

The Analysis Factor for statistical consulting. Funding for this project was provided by a grant from the Morris Animal Foundation (grant #D08ZO-028). This project also was partially supported by a Philip D. Reed, Jr. Fellowship (to M.S.), the Conservation Centers for Species Survival (C2S2) and core support from the Cleveland Metroparks Zoo.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ygcen.2015.05.003>.

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