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Mineral Concentrations in Serum/ Plasma and Liver Tissue of Captive and Free-Ranging Rhinoceros Species

Ellen S. Dierenfeld,[1]* Shirley Atkinson,[2] A. Morrie Craig,[3] Karen C. Walker,[3] W. Jürgen Streich,[4] and Marcus Clauss[5]

[1]*Department of Animal Health and Nutrition, St. Louis Zoo, One Government Drive, St. Louis, Missouri*
[2]*Department of Biology, Muskingum College, New Concord, Ohio*
[3]*College of Veterinary Medicine, Dryden Hall 106, Oregon State University, Corvallis, Oregon*
[4]*Institute of Zoo and Wildlife Research (IZW) Berlin, Germany*
[5]*Institute of Animal Physiology, Physiological Chemistry and Animal Nutrition, Veterinary Faculty, University of Munich, Germany*

Mineral implications in health issues of captive rhinos have received much attention lately. This study was undertaken to establish reference values for the mineral status of rhinos. Serum/plasma samples of free-ranging black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinos and of captive black, white, Indian (*Rhinoceros unicornis*), and Sumatran (*Dicerorhinus sumatrensis*) rhinos, as well as liver tissue samples of captive black, white, and Indian rhinos were analyzed for mineral content. Circulating mineral levels of free-ranging animals were subject to variation according to region. In free-ranging animals, high molybdenum (Mo) values compared to horse normals were striking. Captive animals displayed even higher circulating Mo concentrations. The significance of iron (Fe) overload in captive specimens of the browsing rhinos (black and Sumatran) was confirmed. Hepatic Fe levels increased in blacks with age. Although this Fe overload is suspected to be linked with diets, the data indicate that this is not due solely to an excessive dietary Fe supply. Whereas the grazing species (white and Indian) had high liver copper (Cu) levels, the browsing species had low to marginal liver Cu concentrations. Liver concentrations of K, Mg, Co, and Mo increased with age in captive black rhinos. Additional findings include high circulating Se levels in all rhino species. Future research should be directed at

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*Correspondence to: Ellen S. Dierenfeld, Department of Animal Health and Nutrition, St. Louis Zoo, One Government Drive, St. Louis, MO 63110. E-mail: dierenfeld@stlzoo.org

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investigating factors leading to high Fe levels, and at investigating Cu metabolism in captive rhinoceros species. Zoo Biol 24:51–72, 2005.

Key words: ***Diceros bicornis*; *Ceratotherium simum*; *Rhinoceros unicornis*; *Dicerorhinus sumatrensis*; mineral nutrition; rhinoceros**

INTRODUCTION

Various diseases of captive black rhinoceros have implicated mineral imbalances as an underlying factor. Health syndromes receiving most attention regarding mineral metabolism include those associated with Fe storage disease, and oxidative processes where minerals may be considered essential co-factors [Paglia, 1994; Paglia and Dennis, 1999]. Hemosiderosis has been reported in captive, but not free-ranging, black rhinos [Kock et al., 1992; Montali and Citino, 1993; Paglia et al., 2001]. In captivity, hypophosphatemia has been associated with hemolytic anemia and dermatitis [Kock and Garnier, 1993].

Selected mineral concentrations have been investigated in forages consumed by free-ranging black [Ghebremeskel et al., 1991; Dierenfeld et al., 1995], Sumatran [Lee et al., 1993], and white rhinoceros [Kiefer et al., 2003], but little is known of systemic blood levels, or tissue storage, of these nutrients in any rhino species. Macromineral and trace element ranges in native rhino browses fall into general ranges for horse requirements [Dierenfeld et al., 1995], although sodium and phosphorus may be limiting, the latter particularly in relation to calcium content. Selenium and zinc status in captive black rhinos have also been suggested to be marginal based on limited blood samples [Ghebremeskel et al., 1991], and native browse samples appear to contain low levels of these nutrients [Dierenfeld et al., 1994, 1995].

Dietary mineral interactions, overall health, and nutritional status need to be investigated in detail, particularly in relation to an overall in vivo oxidative status of rhinoceros. This survey study was designed to quantify mineral concentrations in rhinoceros serum/plasma and liver samples in an attempt to establish baseline nutritional assessment criteria for the Rhinocerotidae. We intended to establish mineral levels for free-ranging animals, which should presumably be used as ''normal'' reference for the species, compare these to normal ranges of the domestic model animal, the horse, and then evaluate the status of the captive population. As in earlier studies [Paglia and Dennis, 1999], the comparison between the rhino species was anticipated to be especially revealing.

MATERIALS AND METHODS

Frozen plasma or serum samples (0.5–1 ml aliquots) available from living individuals in North American zoological institutions between 1982 and 2000 were obtained from the AZA (American Zoo and Aquarium Association) Rhinoceros Taxon Advisory Group (TAG) tissue bank, or were shipped directly to the Wildlife Nutrition Laboratory (Wildlife Conservation Society, Bronx, NY). Samples from free-ranging black rhinos were obtained through International Field Veterinary Services, Inc. (Salinas, CA), following rhinoceros translocation operations in Zimbabwe (1998). Blood collection took place after chemical immobilization in the free-ranging animals, whereas a majority of the captive animals were trained for

blood sampling. Liver samples (5–50 g) collected at necropsy, and stored frozen ($-70^{\circ}C$; <10 years) as part of the AZA Rhinoceros TAG research protocol were utilized. Comparative blood and tissue samples obtained from healthy domestic horses were also assayed over the course of this study. All samples were shipped frozen overnight to the Animal Health Diagnostic Laboratory at Oregon State University (OSU AHDL), Corvallis, for mineral determination.

The elemental analysis on the plasma and serum samples was accomplished by using inductively coupled plasma atomic emission spectroscopy (ICP-AES) (JY 2000, Jobin Yvon Inc., Edison, NJ), inductively coupled plasma mass spectroscopy (ICP-MS) (Elan 6100, Perkin-Elmer Corp., Norwalk, NJ) and automated fluorometric determination of selenium (Se) (AAII System with Se cartridge) (Alpkem Corp., Clackamas, OR). Plasma/serum samples analyzed using the ICP-AES were diluted with a protein-precipitation/reducing/internal standard solution. After centrifugation the supernatant was removed for analysis [Melton et al., 1990]. Preparation of the plasma/serum for analysis by the ICP-MS consisted of mixing the sample with a dilute nitric acid solution containing the internal standards. Before analysis the samples were centrifuged [Nuttall et al., 1995]. Liver samples for elemental analysis by ICP-AES and ICP-MS were prepared by adding a measured amount of tissue and nitric acid to a digestion vessel. The sample was then heated overnight in a $100^{\circ}C$ oven. After decomposition the mixture was diluted with an ultra pure water/internal standard solution and centrifuged before analysis [Anderson, 1996; Saito and Saito, 1996]. The instrument calibration, tuning, interference check and internal standard solutions used in the ICP-AES and ICP-MS determinations were supplied by SPEX CertiPrep (Metuchen, NJ) The determination of Se on all plasma/serum and liver samples was conducted using a modification of the method developed by Brown and Watkinson [1977]. The samples were digested by wet-ashing with nitric acid, phosphoric acid and hydrogen peroxide before fluorometric analysis. The Se standard used in this assay was obtained from the National Institute of Standard Technology (NIST) Standard Reference Material (SRM) 3149, Se liver (Gaithersburg, MD). The accuracy and precision of each analysis was established using commercially purchased controls. These materials were obtained from the NIST SRM, 1577b bovine liver and Seronorm Trace Elements, Serum (Nycomed Pharma AS, Oslo, Norway).

Minerals analyzed included calcium (Ca), inorganic phosphorus (P_i), sodium (Na), potassium (K), magnesium (Mg), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), Se, and zinc (Zn). Chloride (Cl) was not analyzed. Additionally, arsenic, cadmium, aluminium, lead, nickel, chromium, vanadium and tin were analyzed in liver samples (results not shown). Available data from the literature was added to the tables; it should be noted, however, that literature data may have been analyzed differently from the current data set.

Differences between black rhinos from different locations were tested by ANOVA and subsequent post-hoc tests. In case of different variances, Dunnett's T3-test was used for the pair-wise comparisons. Differences within species between free-ranging and captive individuals and between free-ranging black and white rhinos, were investigated by *t*-tests for each individual mineral or, if data deviated from the normal distribution, by *U*-test, which is then indicated in the results section. Differences between species serum/plasma levels were tested in captive animals by ANOVA, using species as factor. In three species where n was 3 or less, these were

not compared against each other. Instead of pair-wise post-hoc tests, only a difference between black rhinos and 'other species' was tested (contrast analysis). A potential influence of sex or age was investigated using ANCOVA with sex as a factor and age as covariables. Differences between newborn and adult animals were tested by *t*-test. In three of four species sampled younger animals (age = $<$20 years) were poorly represented, differences between species liver levels were tested in animals $>$20 years of age only to avoid age effects, by ANOVA and post-hoc tests. Again, Dunnett's T3 was used in case of significantly differing variances. All analyses were carried out using the SPSS 9.0 statistical package (SPSS Inc., Chicago, IL). The significance level was set to $\alpha = 0.05$.

RESULTS

Serum/Plasma Samples

Black rhinoceros (Diceros bicornis)

Table 1 provides circulating mineral concentrations in free-ranging black rhinoceros sampled from four locales in Zimbabwe. Mean concentrations of Se in animals from Matusadona, the Midlands, and Chete were below normal for horses. Animals from Matusadona and the Midlands region displayed Fe concentrations within ranges previously reported for captive animals only [Jones, 1979; Paglia and Dennis, 1999]. The only significant differences between regions included Zn and Fe concentrations (Table 1).

When comparing the average circulating mineral levels of free-ranging black rhinos to normal horse ranges (Table 2), a close overlap of ranges can be observed for most minerals. Circulating Ca levels were distinctively higher in the black rhinos, however, and Se levels were lower. Manganese and Mo are not typically requested on clinical serum samples so normal ranges were extracted from the literature.

Circulating mineral concentrations for captive black rhinoceros ($>$1 year of age) are given in Table 3. Captive rhinos displayed lower Mg ($P < 0.001$, $n = 57$), Mn ($P < 0.001$, $n = 40$), and Zn (*U*-test: $P = 0.026$, $n = 57$) concentrations compared to free-ranging animals (data from Table 1, combined from the various locations). The high mean Zn level in captive black rhinos shown in Table 3 was caused by very high levels in three animals from one facility. If these animals were excluded from the comparison, the mean level diminishes to 1.23 ± 0.66 μg/ml, data distribution becomes normal, and free-ranging animals had significantly higher serum Zn levels ($P = 0.035$, $n = 54$). Captive rhinos displayed significantly higher levels of P_i ($P = 0.015$, $n = 56$), Cu ($P < 0.001$, $n = 57$), Mo ($P = 0.003$, $n = 37$), and Se ($P < 0.001$, $n = 57$). The difference in serum Fe levels between free-ranging and captive animals was not significant ($P = 0.074$, $n = 57$). Iron levels for captive black rhinos were within the range reported in the literature, but the mean Fe concentration in free-ranging black rhinos in this study was higher than reported previously. Molybdenum levels in captive animals were considerably higher than normal ranges for domestic horses.

There were no significant differences in circulating mineral levels between the sexes.

Circulating mineral concentrations from neonatal ($<$10 days of age) captive black rhinos are also given in Table 3. Molybdenum was considered higher than

TABLE 1. Serum/plasma mineral concentrations in free-ranging black rhinos (*Diceros bicornis*) from four different locations in Zimbabwe[a]

Area	Ca µg/ml	Pi µg/ml	Na mEq/L	K mEq/L	Mg µg/ml	Cu µg/ml	Fe µg/ml	Mn µg/L	Mo µg/L	Se µg/L	Zn µg/ml
Matusadona ($n=5$)	135.0±16.3	43.6±15.2	142.6±9.4	5.29±0.90	24.0±4.5	1.50±0.41	2.71±0.75	6.52±5.86	4.56±4.25	118.0±21.0	1.49*±0.55
Midlands ($n=7$–8)	124.5±10.3	32.8±9.9	142.0±8.3	4.82±0.40	36.9±11.3	1.69±0.55	2.53*±0.46	7.48±2.71	2.59±1.05	107.9±43.9	1.42±0.34
Chete ($n=10$)	150.7±33.7	28.4±9.7	146.1±13.0	5.09±0.55	27.0±4.8	1.38±0.38	1.71**±0.26	7.95±3.92	7.62±3.91	108.2±36.8	1.67±0.26
Hwange(n = 3–4)	141.8±39.3	39.0±1.0	152.8±10.8	4.33±0.21	27.8±9.2	1.34±0.37	1.77**±0.20	2.82±1.63	6.15±5.67	141.5±41.3	1.13**±0.37

[a]Average±SD. Differing superscripts within columns indicate significant differences.

TABLE 2. Serum/plasma mineral concentrations in free-ranging black rhinos (*Diceros bicornis*) compared with horse values[a]

Source	Ca µg/ml	Pi µg/ml	Na mEq/L	K mEq/L	Cl mEq/L	Mg µg/ml	Cu µg/ml	Fe µg/ml	Mn µg/L	Mo µg/L	Se µg/L	Zn µg/ml
This study ($n=26$–27)	138.7±27.6	33.9±11.5	145.2±10.8	4.93±0.61		29.5±9.0	1.49±0.44	2.15±0.61	6.78±4.00	5.45±4.11	114.8±37.4	1.56±0.43
Kock et al. [1990] ($n=35$–52)	115.0 (96.0–140.0)	37.0 (15.5–62.0)	133.5 (119–151)	4.39 (3.5–6.7)	94 (86–104)	25.6 (12.2–41.3)	—	—	—	—	—	—
Van Heerden et al. [1994] ($n=13$)	114.2±5.6	48.3±9.0	131±3.4	5.09±0.96	93±3.1	25.0±2.7	—	—	—	—	—	—
Keep [1976] ($n=1$)	84.4	—	146.8	7.03	—	22.5	2.4	0.75	—	—	—	—
Paglia and Dennis [1999] ($n=6$)	—		—	—	—	—	—	1.01±0.19	—	—	—	—
Horse normal ranges [OSU AHDL, $n>10$] and Puls [1994], unless noted	100–130	27–50	132–146	2.4–5.6	—	18–35	0.85–2.00	0.8–2.5	>6.0[a], 5.9[b]	20–50[d]	140–250	0.60–1.70

[a]Average±SD. or ranges, respectively.
[b]Normal for bovine serum.
[c]Cieœla [2002], $n=79$.
[d]Stainer and Blackmore [1983].

TABLE 3. Serum/plasma mineral concentrations in adult and newborn captive black rhinos (*Diceros bicornis*)[a]

Source	Ca μg/ml	Pi μg/ml	Na mEq/L	K mEq/L	Cl mEq/L	Mg μg/ml	Cu μg/ml	Fe μg/ml	Mn μg/L	Mo μg/L	Se μg/L	Zn μg/ml
Adults												
This study ($n = 12$–34)	127.0 ±28.3	40.9±11.4	131±7	5±1	—	21.4±5.8	2.06±0.69	2.70±1.11	1.36±1.54	22.1±13.8	195.4±56.1	1.59±1.26
Jones [1979] ($n = 2$–3)	124.3	—	132	5(4–6.2)	94 (93–96)	19.5	1.7 (1.6–1.7)	2.42 (1.30–3.44)	—	—	—	—
Hamilton [1999] ($n = 2$)	116.8±0.28	39.8±2.9	127±3	5.35±0.49	90.7±8.4	27.0±1.7	—	0.69±0.06	—	—	—	—
Vahala [1990] ($n = 1$–3)	114.1±4.6	39.1±12.8	133	5.05	91.5±5.0	20.6±3.7	1.65	2.16	—	—	—	—
Ghebremeskel et al. [1991] ($n = 1$)	—	—	—	—	—	11.5	0.9	—	—	—	30	0.3
Smith et al. [1995] ($n = 40$)	—	—	—	—	—	—	—	6.14±2.00	—	—	—	—
Paglia and Dennis [1999] ($n = 34$)	—	—	—	—	—	—	—	2.47±0.94	—	—	—	—
Newborns												
This study ($n = 2$–3)	130.0±13.5	44.7±9.3	150.7±23.4	4.70±0.53	—	23.7±5.5	3.41±2.23	2.13±1.71	4.58±3.58	12.50±2.12	115.5±116.7	1.19±0.19

[a]Average ± SD or ranges, respectively.

expected compared to normal values for equids. There were no significant differences between circulating mineral levels in newborn and adult animals, and there were no significant correlations of serum/plasma mineral concentrations with age of captive animals except for K, where the correlation was due to two individual outlying values.

Sumatran rhinoceros (*Dicerorhinus sumatrensis*).

Serum/plasma mineral concentrations obtained from captive Sumatran rhinos are presented in Table 4. Average Ca, Mg, K, Cu, and Fe values were above normal equid ranges, whereas Na was low.

Indian rhinoceros (*Rhinoceros unicornis*).

Captive Indian rhino serum/plasma mineral concentrations are also listed in Table 4. The average Zn level was higher than the normal horse range. Although Mo was also above normal horse range, Se was marginally lower.

White rhinoceros (*Ceratotherium simum*).

Table 5 displays Ca and Na concentrations in captive animals that are above the range otherwise reported in the literature for this species; Ca ($P = 0.043$, $n = 8$) and K ($P = 0.050$, $n = 8$) concentrations were also significantly higher than those in free-ranging rhinos in our study. Fe values did not differ significantly between free-ranging and captive animals ($P = 0.795$, $n = 8$) and were intermediate to those reported by others [Jones, 1979; Smith et al., 1995; Paglia and Dennis, 1999]. Insignificant results might, however, also be due to the low power of the test with 6–8 animals only. Compared to horse normals, free-ranging white rhinos had particularly low Na values and high Mo levels.

Interspecies Comparisons

Compared to free-ranging black rhinos, free-ranging white rhinos had significantly higher circulating levels of Se ($P < 0.001$, $n = 32$) and Mo ($P = 0.028$, $n = 30$), and significantly lower levels of Na ($P < 0.001$, $n = 32$), K ($P = 0.010$, $n = 32$), Mg ($P = 0.040$, $n = 32$), Ca ($P = 0.015$, $n = 32$), and Mn ($P = 0.026$, $n = 32$).
Captive black rhinos had significantly higher levels of Fe ($P = 0.034$), K ($P = 0.001$), and Se ($P = 0.046$) than captive Indian rhinos, and lower levels of Mg ($P < 0.001$) than Sumatran rhinos.

Liver Samples (Captive Rhinoceros Samples Only)

Mineral concentrations in liver samples from rhinos > 1 year of age are shown in Table 6, compared to equid normals. Calcium was generally high, and especially so in the black rhino samples. In general, Na was on the low end of the normal horse range. Average Fe levels of all four rhino species exceeded the normal horse range. Although Cu levels were within the horse range for black and Sumatran rhinos, Indian and white rhinos had extremely high liver Cu concentrations. Liver Se concentrations exceeded the normal horse range in both the black and the white rhinos. Other minerals were within expected normals for horses. Data for arsenic, cadmium, aluminium, lead, nickel, chromium, vanadium, and tin did not differ from normal horse levels and are not shown in the table.

TABLE 4. Serum/plasma mineral concentrations in captive Sumatran *(Dicerorhinus sumatrensis)* and Indian rhinos *(Rhinceros unicornis)*[a]

Source	Ca µg/ml	Pi µg/ml	Na mEq/L	K mEq/L	Cl mEq/L	Mg µg/ml	Cu µg/ml	Fe µg/ml	Mn µg/L	Mo µg/L	Se µg/L	Zn µg/ml
Sumatran rhino												
This study ($n=2$–3)	152.3±23.5	58.7±70.5	131±8	6±4	—	36.0±9.17	2.97±0.70	2.86±0.77	1.53±2.65	—	144.7±28.01	1.38±0.56
Indian rhino												
This study ($n=1$–3)	120.7±20.1	40.7±2.52	133.0±9.6	4.14	—	17.3±4.7	2.00±0.49	1.30±0.19	1.89±1.48	5.75±2.04	98.5±2.1	2.35±1.07
Jones [1979] ($n=1$–3)	112.2(108.2–116.2)		134.5 (134–135)	3.7 (3.6–3.8)	88 (86–90)	17.0	1.7 (1.0–2.2)	1.33	—	—	—	—
Paglia and Dennis [1999] ($n=4$)	—	—	—	—	—	—	—	1.23±0.48	—	—	—	—
Flach et al. [2003] ($n=2$–3)	113.4 (111.0–115.8)	26.3 (12.7)	134±2	3.9 (3.7–4.2)	—	—	—	—	—	—	—	—

[a]Average±SD or range, respectively.

TABLE 5. Serum/plasma mineral concentrations in captive and free-ranging white rhinos (*Ceratotherium simum*)[a]

Source	Ca µg/ml	Pi µg/ml	Na mEq/L	K mEq/L	Cl mEq/L	Mg µg/ml	Cu µg/ml	Fe µg/ml	Mn µg/L	Mo µg/L	Se µg/L	Zn µg/ml
Free ranging												
This study ($n=4$–5)	105.6±15.5	35.5±8.7	123.8±4.0	4.15±0.34	—	20.6±4.1	1.16±0.15	1.77±0.66	2.47±2.07	28.34±15.36	200.8±46.1	1.39±0.20
Van Heerden et al. [1985] ($n=20$)	—	—	129.6±4.2	5.4±2.6	94.2±3.1	—	—	—	—	—	—	—
Van Heerden et al. [1994] ($n=6$)	100.2±0.2	—	133.7±3.8	4.31±0.58	95.5±3.3	—	—	—	—	—	—	—
Captive												
This study ($n=1$–3)	139.0±21.7	39.7±12.4	146.7±28.9	4.83±0.45	—	23.7±4.0	2.53±1.30	1.66±0.23	1.65±2.33	19.00	232.0±144.4	1.47±0.33
Jones [1979] ($n=1$–24)	124.3 (116.2–132.3)	—	135.1(133–138)	4.5 (3.6–5.5)	88.8 (86–94)	19.5 (19.5)	1.4 (1.0–2.1)	1.55	—	—	—	—
Seal et al. [1976] ($n=16$)	119.0±0.7	52±0.52	139±5.0	5.1±0.39	95±3.0	—	—	—	—	—	—	—
Smith et al. [1995] ($n=13$)	—	—	—	—	—	—	—	1.87±0.37	—	—	—	—
Paglia and Dennis [1999] ($n=6$)	—	—	—	—	—	—	—	1.34±0.43	—	—	—	—

[a]Average±SD or range, respectively.

TABLE 6. Liver mineral concentrations (wet weight basis) in captive rhinoceros species[a]

	Ca μg/g	Pi μg/g	Na μg/g	K μg/g	Mg μg/g	S μg/g	Co μg/g	Cu μg/g	Fe μg/g	Mn μg/g	Mo μg/g	Se ng/g	Zn μg/g
Adults													
Black Rhino ($n=12$–21)	105±56	3001±606	1203±378	2519±469	186±53	3092±491	0.63±0.73	6±4	4636±5473	3.22±4.88	3.99±5.03	2283±1694	79±42
Sumatran Rhino ($n=2$)	69±8	3550±354	960±198	2600±849	230±28	2900±0	0.92±0.54	5±0	4960±6279	BDL	2.55±1.91	423±30	59±1
Indian Rhino ($n=2$–4)	94±25	2700±990	1303±467	2322±260	175±21	3000±283	0.14±0.24	170±296	833±312	2.84±0.50	0.81±0.46	877±520	91±57
White Rhino ($n=5$–6)	61±15	3060±391	958±181	2608±418	187±38	2420±164	0.37±0.05	83±88	530±390	0.47±0.53	0.85±0.50	1656±1501	78±77
Horse Normal Ranges (this study and Puls [1994])	40–60	2600–4000	900–1800	2000–4000	130–200	—	0.02–1.0	4.0–7.5	100–300	1.0–6.0	0.5–6.0	300–1000	40–125
Newborns													
Black rhino ($n=2$–4)	120±38	2733±839	1667±306	2033±208	164±31	2667±462	0.28±0.21	315±246	602±197	0.18±0.36	0.30±0.60	644±170	186±126
Indian rhino ($n=2$)	87±16	2250±212	1300±0	2100±141	145±7	2550±71	0.44±0.01	245±49	400±170	0.00±0.00	0.00±0.00	318±45	101±55

[a]North American zoos; BDL = below detection limit, below detection levels. Average ± SD.

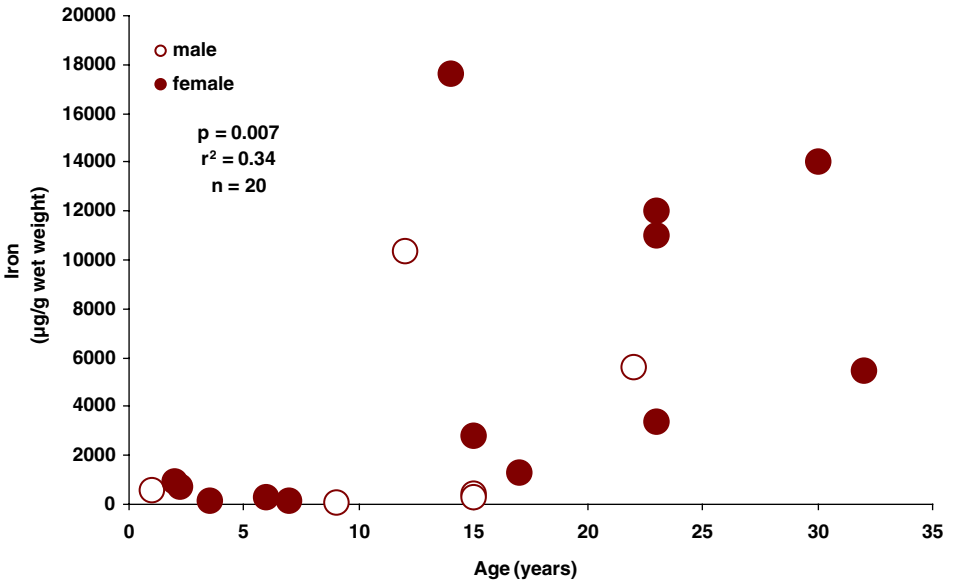

Fig. 1. Iron levels in livers of captive black rhinos (*Diceros bicornis*) correlated with age.

Livers from neonates (<10 days of age) or stillborns are also found in Table 6. Samples from these young contained significantly lower Fe ($P=0.003$, $n=25$), Mo ($P=0.004$, $n=25$), and cadmium ($P<0.001$, $n=22$; [data not shown]), and significantly higher Cu (U-test: $P=0.001$, $n=25$) concentrations compared to adult animals. There were no significant differences between neonates and adults in the Indian rhino, which might, however, be due partially to small sample size (4–6 animals).

There were no significant differences in liver mineral concentrations between the sexes in black rhinos. There were significant correlations between age and hepatic K ($P=0.039$, $n=19$), Mg ($P<0.001$, $n=20$), Co ($P=0.038$, $n=20$), Fe (0.012, $n=20$), and Mo ($P=0.005$, $n=20$) in black rhinos (Figs. 1,2).

Black rhinos (>20 years of age) had significantly higher hepatic levels of Fe and Mo than white ($P=0.005$ and $P=0.029$) or Indian ($P=0.006$ and $P=0.035$) rhinos, respectively. No other significant differences between any species could be detected. The hepatic Fe value for the other browsing species, the Sumatran rhino, was of the same scope as the black rhino value but could not be compared statistically due to low n.

DISCUSSION

By necessity, a survey of this scope depends on samples taken by a multitude of individuals, and therefore a consistent quality of the material submitted cannot be guaranteed. Ideally, in such a study the degree of hemolysis of plasma/serum samples should be monitored by analyzing the hemoglobin content of the sample. In this study, hemolysis was only assessed visually, and a total of six samples were considered 'hemolytic.' The liver samples were from necropsied animals that had

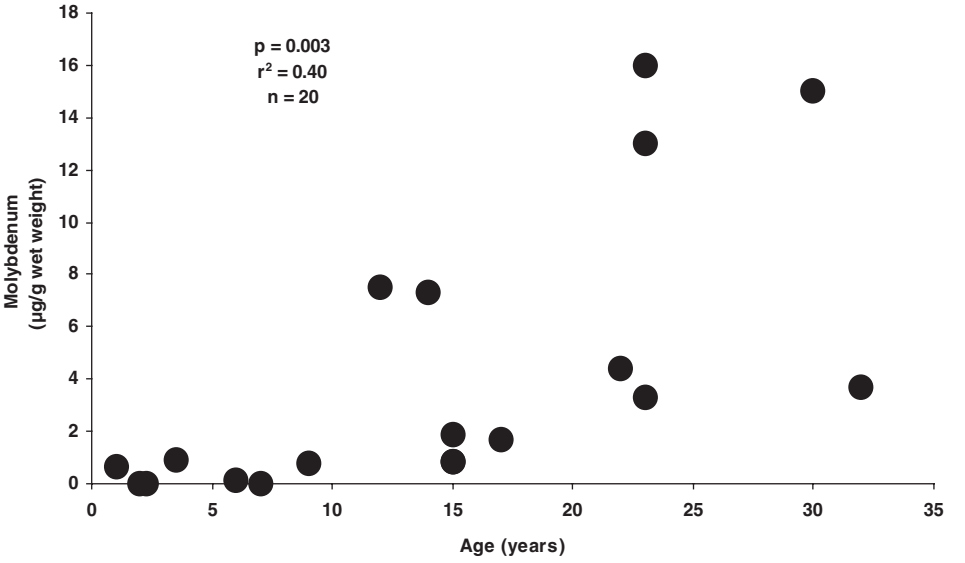

Fig. 2. Molybdenum levels in livers of captive black rhinos (*Diceros bicornis*) correlated with age.

died due to a variety of causes, and the mineral concentrations measured might therefore not represent 'normal' values of healthy animals. Ideally, liver mineral concentrations should be determined on a dry matter basis to minimize differences due to water losses during frozen storage, and histopathological assessment should accompany mineral determination. Direct correlation of liver and serum/plasma values could not be made because samples from the same individuals were unavailable. Literature data used for comparative purposes may have been analyzed differently from the current data set and differences may be an artifact of method of analysis. The data determined in the course of this study must be interpreted with caution for all these reasons.

Calcium and Phosphorus

Calcium levels in livers were generally high. Although white rhino samples were just on the upper range of horse standard values, black rhinos had particularly high liver Ca concentrations. A potentially pathological significance of this finding is unknown. A dietary cause, however, can be suspected: rhinos, like equids, tapirs and elephants [Schryver et al., 1983], and lagomorphs [Cheeke and Amberg, 1973], probably do not regulate Ca uptake in the gut as man and other animals do, but absorb it in great quantities, excreting any unnecessary surplus via the kidneys. Differences in dietary Ca should be reflected in differences of circulating Ca levels. Free-ranging black rhinos, for example, have significantly higher circulating Ca levels than free-ranging white rhinos, probably because black rhino natural forage (browse) generally contains higher concentrations of Ca than white rhino natural forage (grass) [Robbins, 1993; Dierenfeld et al., 1995]. Black rhinos are traditionally fed larger amounts of Ca-rich alfalfa hay, in contrast to white and Indian rhinos, who traditionally receive grass hay in many institutions. The permanent high dietary

Ca supply via alfalfa might lead, over time, to higher Ca deposits in liver tissue. Kiefer et al. [2003] demonstrated higher Ca concentrations in a hay fed to white rhinos in a European zoo compared to Ca concentrations in natural South African forage. This discrepancy could explain the generally higher circulating Ca in captive compared to free-ranging white rhinos. Additionally, captive animals are likely to receive, in addition to the minerals supplied via forage, special mineral supplementation products. With respect to potential calcinosis and Ca urolithiasis the efforts necessary to clean Ca-rich urine deposits from walls and floors of rhino enclosures, the Ca supplementation regime in captivity should be re-evaluated.

Hypophosphatemia has been observed in black rhinos during hemolytic crises [Kock and Garnier, 1993; Paglia, 1994]. Our data, however, do not suggest that captive rhinos of any species are generally P_i marginal or deficient, which implies that the problems are not of a general, dietary origin. In contrast to Kock et al. [1990] we did not find a correlation with P_i and age, nor with Ca and P_i and sex.

Sodium, Potassium, and Chloride

It has been noted earlier [Van Heerden et al., 1994] that rhinos had low serum levels of Na and Cl; for Na, this finding is supported by our data. Sodium was reported to be in limited to marginal supply in browse consumed by black [Ghebremeskel et al., 1991; Dierenfeld et al., 1995] and Sumatran rhinos [Dierenfeld et al., 1994], and Na is generally a limiting factor for many wild herbivores [Robbins, 1993]. The relatively low Na (and concurrently Cl) values displayed by rhinos might be an adaptation to a habitat where this mineral is limited. Potassium is usually abundant in any forage material [Robbins, 1993]. The correlation with age suggests that it is accumulated in the liver over time. There is no reason, however, to suspect that this is potentially problematic.

Magnesium

Magnesium concentrations in the liver were high in all rhino species and exceeded the normal horse range for Sumatran rhinos. There was a correlation of liver Mg levels and age suggesting an accumulation of Mg over time. Magnesium was reported to be in marginal supply in black rhino browse species [Dierenfeld et al., 1995]. For captive animals, Mg supply seems adequate to high. As no pathological observations have been reported that could be related to an oversupply of Mg this is unlikely to warrant special attention.

Cobalt

Although Co was not measured in the serum/plasma, its content in liver tissue was within the horse normal range, with the browsing rhino species attaining the higher levels. In black rhinos, higher Co levels were found in older animals, and the correlation with age was significant. Cobalt and Fe share a common intestinal mucosal transport pathway, and have been shown to function as antagonists at the absorptive level [Smith, 1987]. Iron deposition in the liver also increases with age in captive rhinos [Paglia and Dennis, 1999]; one might expect the high Co to interfere with Fe absorption. In idiopathic hemochromatosis in humans, however, Co absorption was increased in parallel to Fe absorption, and in patients with Fe overload, Co absorption was not reduced in contrast to Fe absorption [Valberg et al., 1969]. This suggests that the Fe overload in rhinos is not simply a consequence of

high dietary Fe levels, or one might have expected reduced Co absorption [Smith, 1987]. In contrast to ruminants, Co deficiency has not been demonstrated in horses [Smith, 1987]. From our limited data, the dietary supply seems adequate for rhinoceros species.

Copper

In our sample, serum/plasma Cu levels in captive rhinos (both black and white) were higher than values for free-ranging animals. Serum/plasma Cu values were above the horse normal range, and this was a common trend for all four rhino species. The species differed significantly in their liver Cu content. Although the browsing species had average liver Cu values within the horse normal range, the Indian and the white rhino had values several magnitudes higher. In contrast, when Jones [1979] compared Cu contents of liver tissue, he found no difference between one black and four white rhinos. Howard [1964] gave an average Cu liver content for six free-ranging black rhinos in Kenya of 28.7 μg/g dry matter (SD = 3.5; range = 20–43). Assuming a liver dry matter content of 25–30%, this would translate into 7–9.5 μg/g wet weight (WW) (range = 5–14). This is within the upper normal range for horses and compares well to our average and range for captive rhinos of 6 and 3–17 μg/g WW, respectively. It should be noted, however, that in our sample, six black rhinos had liver Cu levels of 3 μg/g WW, which is below the level of 3.5 Puls [1994] gives as the threshold below which a deficiency might be suspected.

The first striking discrepancy in our findings is the fact that the these enormous differences in liver Cu content are not reflected in serum/plasma Cu content. Samples did not originate from the same individuals. Whereas there is a good correlation between Cu levels in serum and liver in ruminants, i.e., Cu status can be assessed sufficiently accurately by serum Cu [Vermunt and West, 1994], there is no reliable correlation between the two variables in equines [Smith et al., 1975; Cymbaluk and Christensen, 1986; Suttle et al., 1996]. This could mean that serum/plasma Cu is not a reliable indicator of Cu status in rhinos.

Copper deficiency is a cause for excessive liver Fe storage in most animals [Brewer, 1987]. High liver Cu levels have been found in human hemochromatosis [Davis and Mertz, 1987], and Cu toxicosis leads to a hemolytic crisis in many animals [Brewer, 1987]. This means that one would expect either significantly high or low liver Cu levels in those species that do have problems associated with Fe and hemolysis (e.g., black rhinos). There were no significant correlations between Fe and Cu levels in serum or in liver in this study.

The fact that it is the grazing species, those who do not have Fe problems, that have liver Cu levels deviating from the normal range, is therefore a surprising finding. There are species differences in the susceptibility to Cu toxicosis, with equids being highly resistant [Smith et al., 1975; Brewer, 1987]. There are no reports of clinical signs associated with Cu toxicosis in captive white and Indian rhinos. In one study, the natural diet of free-ranging white rhinos contained 4–6 mg Cu/kg DM only [Kiefer et al., 2003] (horse recommendation 10 mg/kg DM [National Research Council, 1989]). If one assumes that these animals had to adapt to diets of low Cu content, a particular storage of this mineral could be expected and explain the high liver Cu values determined in this study.

In black rhinos, although the average Cu liver content was within the normal horse range, six animals were deficient according to horse standards. It was shown

that the diet consumed by Sumatran rhinos in a North American facility, consisting of pellets, hay, produce, and browse, was low in Cu (6.9–8.3 mg/kg DM) compared to horse dietary requirement recommendations (10 mg/kg DM) [National Research Council, 1989], although potential contributions from an available trace mineralized salt block were not incorporated into the diet analysis [Dierenfeld et al., 2000]. Similarly, another diet reported for black rhinos [Spala and Hradecky, 1993] contained a marginal 7.3–9.3 mg Cu/kg DM (pellets, oats, hay). Therefore, a marginal dietary Cu supply in individual browsing rhinos cannot be excluded.

The influence of a potential Cu deficiency in the occurrence of encephalomalacia in newborn black rhinos has been speculated upon [Paglia et al., 2001]. In horses, however, clinical signs of degenerative myeloencephalopathy were not correlated with either low plasma or liver Cu concentrations [Mayhew et al., 1978; Dill et al., 1989]. In our sample, newborns had higher liver Cu contents than older animals, in accordance with data from other species [Davis and Mertz, 1987], but the high variation precluded statistical significance. The high Fe load of captive black rhinos, and the ensuing oxidative stress [Paglia and Dennis, 1999], might necessitate higher rates of Cu metabolism as Cu is an important part of several antioxidant enzyme systems [Johnson and Fischer, 1992]. In rats, Cu deficiency increases the susceptibility of tissues to Fe-induced lipid peroxidation [Rayssiguier et al., 1993], and Cu deficiency is correlated with an increase in red blood cell lipid peroxidation and a reduction in erythrocyte survival [Jain and Williams, 1988]. The occurrence of several disease complexes in black rhinos that are potentially linked to oxidative stress due to the high Fe load [Paglia and Dennis, 1999] might indicate a special requirement for Cu in these animals, not as an evolutionarily developed peculiarity, but as a consequence of Fe overload in captivity. Feeding a marginal Cu diet to rats significantly attenuated phagocytic cell activity in vitro, while altering, only marginally, the concentrations of serum and hepatic Cu [Babu and Failla, 1990a,b]. The experimental design showed a decrease in the antifungal capacity of the immune system. Fungal pneumonia has been repeatedly observed in black rhinos [Weber and Miller, 1996]. These findings should not lead to the conclusion that black rhinos should receive special Cu supplementation until our findings have been confirmed and the Cu metabolism of rhinos is investigated in detail. Specifically, the Cu status of living individuals should be assessed in a survey that not only measures plasma Cu, a weak predictor of Cu status, but ceruloplasmin and erythrocyte superoxide dismutase before and after parenteral Cu supplementation [Danks, 1981], as well as other interacting minerals such as Mo and Sulfur. Such assays were conducted for the evaluation of Cu status in koalas (*Phascolarctos cinereus*) [Thomas et al., 1986], as well as other interacting minerals such as Mo and sulfur.

Iron

Our results confirm the importance of high Fe loads in captive black rhinos with their probable consequence of excessive oxidative stress [Paglia and Dennis, 1999; Paglia et al., 2001]. Differences in serum Fe between free and captive animals were not as distinct in our sample as cited previously [Paglia and Dennis, 1999], and the high values reported for captive animals [Smith et al., 1995] were not reached in our data set. In contrast, some free-ranging animals displayed relatively high serum Fe values, indicating that Fe levels vary according to habitat. When reviewing the literature, the low Fe values from Hamilton [1999] are surprising and warrant

explanation. Paglia and Dennis [1999] found average values for free-ranging (Table 2) and boma (1.44 ± 0.44 μg/ml, $n = 6$) animals that were lower than values found in free-ranging animals in this study. These results underline the findings from Table 1 (e.g., circulating Fe levels are likely to vary with region and possibly with age in free-living animals). Paglia and Dennis found that levels in animals imported recently (2.43 ± 1.03, $n = 9$) only slightly exceeded those of free-ranging animals found in our study. Although possible influences of gender and genetic predisposition to Fe storage problems were speculated upon [Paglia et al., 2001], we did not find a significant difference of serum Fe load and liver Fe load between the sexes in black rhinos. Clinical signs of Fe storage have been shown to increase with age in captivity [Paglia and Dennis, 1999] and the data confirmed this. Newborn black rhinos had lower Fe levels in serum/plasma and liver than older specimens. Liver Fe increased significantly with age; similar to another study [Smith et al., 1995]. There was, however, no significant increase in circulating Fe levels with age. It has been stated repeatedly that for a proper assessment of Fe status, serum ferritin, and transferrin saturation indices should be used rather than only serum Fe [Paglia and Dennis, 1999].

The serum/plasma and the liver data show particularly high Fe levels in the browsing rhino species, and lower Fe levels in the Indian and white rhino. These results are in accord with observations [Paglia and Dennis, 1999] that reported circulating Fe levels of 2.22 (± 0.65, $n = 3$) for Sumatran rhinos, and support the notion that it is the dietary specialization of the browsing animals that leads to the Fe storage problem in captivity. Further evidence in another browsing species guild, the tapirs (*Tapirus* spp.) add weight to this hypothesis [Paglia et al., 2000], and it has been proposed that Fe overload is common to monogastric, browsing species in general [Clauss et al., 2002].

Manganese

Only free-ranging black rhinos displayed serum/plasma Mn concentrations that would be considered within an expected normal range for equids (low 7.8 μg/L to a mean of 15.9 μg/L [Cieśla, 2002]). Variation of Mn serum/plasma values was generally very high, even in free-ranging black rhino. Liver concentrations were also low in captive black and white rhinos and showed very large variation. This could suggest an inconsistent and, at least for certain individuals, a marginal to deficient supply of this mineral. Deficiency of Mn can result in depressed reproductive function (defective ovulation, testicular degeneration, stillbirths, or early death), depressed oxidative enzyme (SOD) activities, and defects in carbohydrate and lipid metabolism, the latter leading to increased lipid peroxidation in cell membranes [Hurley and Keen, 1987]. None of these effects have been investigated in detail in any rhino species.

Molybdenum

The Mo values for captive black and white rhinos, as well as free-ranging whites, were within a reported normal range for equids [Stainer and Blackmore, 1983] whereas free-ranging blacks, newborns, and Indian rhinos were variable but low. This suggests habitat/dietary influences may have more impact than species per se. Molybdenosis is associated with low Cu availability and Cu deficiency in ruminants [Mills and Davis, 1987], and has been suspected to be the cause of clinical Cu deficiency in horses [Walsh and O'Moore, 1953]. There are large species

differences in the tolerance to high dietary Mo levels, with horses being very tolerant [Mills and Davis, 1987]. Oral doses of Mo did not influence Cu serum or plasma levels in horses [Cymbaluk et al., 1981; Strickland et al., 1987], but as stated above, serum/plasma Cu values are not the most reliable measure of Cu status. It was demonstrated that plasma Mo levels in horses responded to dietary changes, there was no correlation between plasma Cu and Mo levels (nor was there in our data set), and dietary Mo reduced Cu availability [Cymbaluk et al., 1981]. Although there is little evidence of overtly excess Mo from the rhino serum/plasma data, dietary Mo content might, given the increased oxidative stress in captivity, contribute to the probability of Cu deficiency. Mo has been reported to accumulate in the equine liver [Moore, 1958], and in rabbits, high dietary Mo induced increased concentrations of liver Fe compared to a control group [Titame et al., 1997]. Newborn rhinos did not have high Mo liver contents, but in black rhinos, liver Mo concentrations increased significantly with age, indicating an accumulation process. Dietary levels of Mo should be measured with respect to Cu concentrations, and potential mineral interactions taken into account in determination of nutrient status.

Selenium

Native browses eaten by black rhinos in at least one location [Ghebremeskel et al., 1991], as well as those consumed by Sumatran rhinos [Dierenfeld et al., 1994], have been shown to be low in Se content. Although lower Se values in food of captive black rhinos as compared to forage from the wild were found in one study [Ghebremeskel et al., 1991], diets consumed by captive Sumatran rhinos were not low [Dierenfeld et al., 2000], and our physiological data do not suggest a deficiency. Rather, Se levels were surprisingly high in the livers of both black and white rhinos. We conclude that Se levels in feeds offered to both black and white rhinos may be high relative to dietary requirements, with resulting tissue accumulation. "Toxic" doses of Se lead to equine serum levels of 280 μg/l and higher [Witte et al., 1993]. These reported toxic ranges were higher than the averages observed in our data, but two rhinos had excessive levels by these criteria. Other authors [Detlef et al., 1995] report selenosis in horses where the serum levels range between 199–365 μg/L. In this case, most of the captive rhinos would fall in the lower end of the suspected toxicosis range. Puls [1994] considers values 350–1,000 μg/L as elevated. Regional differences in feeds and mineral interactions need to be taken into account in assessing status.

Many areas in North America have soils that are known to contain high levels of Se [Trelease and Beath, 1949], and forages grown on those soils can accumulate this mineral. Thus, alfalfa as a passive Se accumulator has repeatedly been reported to be high in Se [Trelease and Beath, 1949; Mayland et al., 1989; Witte et al., 1993]. Mineral assay of forages and other ingredients consumed is recommended when evaluating diets. A more moderate supply of this mineral, with respect to the high circulating and hepatic concentrations measured in this study, might be appropriate.

Excessive levels of dietary Se have been shown to trigger hair problems, lameness, and hoof sloughing [Witte et al., 1993], but no effect on fertility was seen in horses. In pronghorn antelope (*Antilocapra americana*), in the absence of overt disease, high Se tissue levels were correlated with a decreased primary antibody response to an egg-albumin challenge [Raisbeck et al., 1996], and it was concluded that chronic selenosis leads to a subclinical immune impairment. Interactions

between dietary Se and Cu have been identified in horses [Stowe, 1980], with the potential for Se toxicosis increasing with decreasing dietary Cu.

Zinc

Although lower Zn values were found in food of captive black rhinos as compared to forage from the wild [Ghebremeskel et al., 1991], and marginal Zn levels in wild rhino forage compared to horse requirements [Dierenfeld et al., 1995; Kiefer et al., 2003], data does not suggest a deficiency, in the wild or in captivity. The lowest Zn serum concentration in our sample of captive black rhinos was still twice as high as the low level reported by Ghebemeskel et al. [1991]. In horses, animals fed in stables had higher Zn levels in serum than animals at pasture [Stubley et al., 1983]. In our sample serum/plasma Zn levels were comparable between free and captive animals and, except for the high levels found in captive Indian rhino, within the normal horse range. Similarly, liver Zn concentrations were within the normal horse range.

Dietary Iron and the Chelator/Tannin Theory

Captive browsing rhinos display signs of Fe overload, which is suspected to be of dietary origin. Several nutrients such as Cu [Storey and Greger, 1987], Zn, P [Morris, 1987], and Co [Smith, 1987], have been shown to be reduced in availability in high Fe diets, and generally do not seem limiting in our sample in any rhino species (with the possible exception of Cu only). This suggests that although Fe overload in rhinos may very well be linked with diets, it is probably not caused solely by a relative excess of Fe in captive diets. Our data suggest that the relative proportions of minerals in captive diets are adequate, their absolute amounts notwithstanding, with the possible exception of Cu. Other factors must be responsible for the Fe overload in the browsing species. It has been hypothesized repeatedly that captive browsing animals, the browsing rhinos included, can suffer from Fe overload due to a relative "lack of tannins" in captive diets [Spelman et al., 1989; Paglia and Dennis, 1999]. This theory is based on the observation that tannins/polyphenols reduced Fe availability in numerous studies [Disler et al., 1975; Gillooly et al., 1983; Jansman et al., 1993]. The effect of dietary tannin supplements and of low-Fe diets on browsing rhinos should be investigated.

CONCLUSIONS

This study presents a comprehensive body of reference data for the mineral evaluation in rhinoceros species. The assumption that horse requirements can be used for comparative purposes was confirmed, although rhinos have lower circulating Na and seem to have higher circulating Ca concentrations compared to horses. Captive rhinos, but not free-ranging, also display relatively high circulating Se levels. Captive specimens of the browsing rhino species display increased Fe levels both in serum and liver tissue. A comparative evaluation of mineral data suggests that although this Fe overload might be of dietary origin, an unduly high dietary Fe provision can be ruled out as the causative factor. At the same time, captive specimens of this group seem to be prone to a low Cu status that warrants further investigation, particularly with respect to known mineral interactions in other species

(Mo, Se). Differences in tannin content between captive diets and native forages could contribute to the high Fe/low Cu phenomenon.

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