CASE STUDY: IRON IN BLACK RHINOCEROS DIETS: THE IMPACT OF PASTURE

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Abstract
Over the course of a year, serum ferritin and iron in a black rhinoceros (*Diceros bicornis*) increased over time, despite a prescribed diet low in iron. To identify additional sources of iron intake, vegetation in the enclosure was analyzed for iron content. Iron concentrations in enclosure samples averaged 736 ppm, well in excess of maximum recommended total-diet concentrations of 300 ppm. The rhinoceros consumes pasture grass in the enclosure, which may be leading to high iron intake and subsequent internal accumulation. We recommend that institutions holding iron-sensitive rhinoceros species test any vegetation available to the animals, and not only prescribed feeds.

Introduction
A 24-year-old male black rhinoceros (*Diceros bicornis*) was received into the Denver Zoo's collection in February 2016. Due to known concerns over iron overload disorder (IOD) in members of this species under managed care, the animal's iron status was monitored on a routine basis. A review of the prescribed diet in October 2016 showed total-diet iron concentrations below the recommended maximum; however, his serum ferritin and iron values continued to increase. Because the animal's enclosure was vacant for several months prior to his arrival, a large amount of standing vegetation was available, and the animal readily grazed all areas of his enclosure once given access. Thus, we investigated enclosure vegetation and soil as potential sources of excess iron intake.

IOD is characterized by chronic accumulation of iron in the body leading to increased morbidity, including compromised immune system, impaired antioxidant capacity, lethargy, liver damage, and in rare cases, acute death (Dennis et al., 2007; Khan et al., 2007; Paglia and Radcliffe, 2000; Sullivan et al., 2016). In human-managed care, the disease prevails in the black and Sumatran (*Dicerorhinus sumatrensis*) rhinoceros (Candra et al., 2012; Claus and Paglia, 2012; Sullivan et al., 2016). Wild rhinoceroses, however, do not appear to suffer from IOD. Given the current conservation status of rhinoceros populations (all species are vulnerable, near threatened, or critically endangered), preventing and treating IOD to maintain healthy managed populations has become increasingly important.

Materials & Methods
Analysis of the prescribed diet included manufacturer-reported nutrient concentrations in commercial feeds (Mazuri Exotic Animal Nutrition, St. Louis, MO), USDA nutrient data for produce (USDA Food Composition Database, Release 28, 2015), and laboratory analysis of *Ficus* browse as well as local alfalfa and timothy hay (Dairy One, Inc. Forage Testing Laboratory, Ithaca, New York). These values were used to mathematically calculate total-diet iron concentration using Microsoft Excel. Diet review occurred in October 2016, with modifications to the diet immediately following.

Serum samples were collected as part of the animal's initial quarantine examination in March 2016, and every 2 to 6 months thereafter as part of his routine clinical follow-up. Samples were analyzed for complete cell count, serum biochemistry, serum iron, serum ferritin, and total iron binding capacity and compared with published findings and recommendations (Candra et al., 2012; Miller et al., 2016; Paglia and Radcliffe, 2000; Paglia and Tsu, 2012).

Grass in the black rhinoceros enclosure was sampled in November 2016 and again in June 2017. At the November sampling period, 2 replicate samples were collected from the upper and lower section of the enclosure, respectively, and replicate samples were averaged together. Samples from leaves and seed pods that fall into his enclosure were also collected during the November collection period. During the June sampling period, grass samples were also collected from several other enclosures in the zoo, again with 2 replicates per enclosure, to assess institution-wide variation in pasture nutrient composition. Grass and leaf samples were dried for 24h at 100°C in a gravity convection oven (Thermo Scientific Heratherm OGS60 Lab Oven) and then sent to a commercial laboratory (Dairy One, Inc. Forage Testing Laboratory, Ithaca, New York) for proximate and mineral analysis.

Enclosure soil was also sampled during the November sampling period from three distinct locations in the black rhinoceros yard. Samples were sent to a laboratory (Colorado State University Soil, Water, and Plant Testing Laboratory, Fort Collins, CO 80523) for mineral analysis.

Results & Discussion

The total prescribed diet fed from March to October 2016 provided 163.2 ppm iron. Following diet review and modification, dietary iron concentration fell slightly to 161.4 ppm (Table 1). These values do exceed the recommended range of 50-100 ppm; however, they fall well below the maximum recommended limit of 300 ppm iron in the diet (Clauss et al., 2012). Although alfalfa hay has been identified as a potential contributor of iron to commercial feeds and ad-libitum consumption (Koutsos et al., 2016), the Colorado-grown alfalfa provided to the animal between March and October 2016 contained only 142 ppm iron.

Despite seemingly low dietary concentrations, serum ferritin and iron concentrations continued to rise (Figure 1), from initial values of 455 ng/mL and 162 ug/dL, respectively, in March 2016, to 1559 ng/mL and 279 ug/dL, respectively in February 2017. Serum transferrin saturation $\left(\frac{serum\ iron}{total\ iron\ binding\ capacity}\right)$ increased from 44% in March 2016 to 82% in February 2017 (Figure 2). Additionally, serum concentrations in April 2017 fell to 1125 ng/mL ferritin and 243 ug/dL iron with 71% transferrin saturation.

The winter of 2016-2017 was warm in Denver, particularly in February, with temperatures between 60-80° F, allowing the animal greater outdoor access and time to graze. Temperatures in April returned to more seasonable ranges (monthly average of 48°F), causing the animal to spend more time indoors and resulting in pasture senescence. A rise in all values analyzed in February, followed by a fall in these values in April, seems consistent with periods of time when the animal spent more and less time grazing, respectively.

In addition to changes in iron status, serum ferritin can respond to systemic inflammation (Biemond et al., 1984; Kalantar-Zadeh et al., 2004); thus total-health status is important to consider when interpreting the results of serum iron panels. This individual has a history of severe dental tartar accumulation and oral eosinophilic granulomas, which could cause low-grade inflammation (Pessier et al., 2004). However, these issues were present when the animal arrived at Denver. The dental disease is stable, and the incidence of oral eosinophilic granulomas has been sporadic, with no reported occurrences in February. Thus, it seems unlikely that changes in dental or oral health caused the spike in serum ferritin in February 2017.

Analysis of exhibit vegetation sampled in November 2016 revealed iron concentration ranging from 497 to 1,385 ppm iron (Table 2). Grass sampled from the rhinoceros enclosure in June 2017 contained even higher levels of iron at 2110 and 3020 ppm in the upper and lower yards, respectively. Iron content in other pastures was dissimilar to that of the rhinoceros enclosure with the exception of the bongo enclosure, which had an average iron content of 2855 (Table 2). The analysis shows that high iron grass is not an institution-wide problem. The soil in these other enclosures has not yet been analyzed.

Items consumed by black rhinoceroses in their native habitat average 82 ppm iron with a range of 12 to 215 ppm, whereas the temperate browse, grass hay, and alfalfa hay often used in European and North American institutions average 120, 180, and 129 ppm iron, respectively (Clauss and Hatt, 2006). However, iron content in cultivated feeds can vary widely, reaching concentrations as high as 2,599 ppm in some forage (Clauss et al., 2012). Helary et al. (2012) found that total-diet iron for wild black rhinoceros ranged from 49.5 ± 10.9 to 175 ± 31.5 ppm. In contrast, human-provided diets average 374 ± 224 ppm iron (Clauss et al., 2007a).

Enclosure substrate presents another potential source of iron ingestion, and soil composition contributes in turn to standing pasture nutrient concentrations. However, the soil in this animal's enclosure contains an average of 8.33 ± 5.01 ppm iron (Table 1), reported by the analyzing laboratory as low to adequate for growth of temperate monocots. The lower exhibit yard, with the highest soil iron concentration (14.1 ppm), also contained 2.5% organic matter, considered high in comparison with most soils (<2%; fertile soil contains >2.8% organic matter; Petit, 2004). The upper exhibit yard, which has a lower iron content in its grass, has what is considered a low amount of organic matter (2.1%). Humus comprises 65-75% of organic matter (Petit, 2004), and plays an important role in anion and cation exchange, including iron. Iron-humate complexes can be used as a source of iron for plants and are actually used to treat iron-deficient vegetation. Some studies suggest that iron-humate complexes can contribute to iron absorption when soil iron levels are low (Varanini and Pinton, 2006). We suggest that increased organic matter in the lower yard may be responsible for greater iron accumulation in growing pasture.

Plant leaves accumulate iron, with 80 to 90% stored in the chloroplasts. Along with roots, leaves represent a major iron-storage reservoir for many plant species, including grasses, for which the blade comprises a significant portion of the leaf and of the plant as a whole (Briat et al., 2006; Terry & Abadía 1986). Wild, untreated *Cynodon dactylon* (scotch grass), for example, was found to have iron levels ranging from 234 ppm in the leaves to 10,456 ppm iron in the roots (Franco et al., 2013; Landschoot, 2007). When presented with excess iron, some grasses such as *Setaria parviflora* (marsh bristlegrass) and *Paspalum urvillei* (Vasey's grass), can accumulate iron at levels above those considered critically toxic to plants. Although these species accumulate most iron in the roots, shoots also displayed potentially phytotoxic iron concentrations (de Araújo, 2014). This pattern has also been noted in some rice-producing grass species (Dobermann, 2000; Liu et al., 2008).

Denver Zoo's black rhinoceros exhibit contains a cool-season grass mixture of ryegrass, orchardgrass, wheat, and brome. Cool-season grasses generally range from 50-150 ppm in iron concentration, though this varies greatly depending on factors such as location, care, and water. Although the iron concentration in this enclosure's soil is considered low, cool-seasons grasses only require >4 ppm iron; thus, current concentrations of 5 to 14 ppm are more than adequate for these grass species. Given the aforementioned ability for some grasses to accumulate iron when presented with soils in excess of their requirement, iron accumulation may likewise be possible in the current situation (Mayland & Wilkinson, 1996). Denver Zoo's rhinoceros pasture appears to contain a perfect storm of grasses with a low iron requirement and soil with more than adequate iron content and high organic matter. The grass species present in the other species' enclosures is under investigation, and we have not yet analyzed all parameters in those exhibits; however, it is possible that the differences in iron content between the black rhino and other enclosures could result from differing grass species.

It is important to note that we have not fully quantified all sources of dietary iron for this animal. We have not yet analyzed all browse species fed, determined iron concentration in drinking water, nor evaluated seasonal changes in exhibit pasture. Furthermore, dry-matter pasture consumption and the animal's time spent grazing have not yet been quantified, so we cannot yet determine the exact degree to which standing forage in the enclosure impacts total-iron ingestion. However, browse and water sources have been consistent year round, and pasture consumption (or lack thereof) is the one variable identified to date which has changed over time.

Conclusion

Few clinical treatments for IOD exist. Application of iron chelators and phlebotomy, though effective, burden time and financial resources, and can involve risks (Sullivan et al. 2015, Sullivan et al., 2016). Arguably, the most practical (albeit difficult), solution for IOD incidence is to prevent it altogether (Clauss and Paglia, 2012). Since diet can be a major contributor (Candra et al., 2012; Clauss and Hatt, 2006; Helary et al., 2012; Ruetten et al., 2009), many institutions that care for rhinoceroses provide low-iron diets, and multiple investigations have explored iron ingestion from prescribed diets and water. However, few published studies have evaluated available vegetation in enclosures. While some rhinoceroses are managed on dry lot, many have accessible planted grass, trees, and shrubs.

Our analysis revealed high levels of iron in the vegetation accessible to Denver Zoo's black rhinoceros. The animal has been observed consuming this vegetation, and it is a possible source of excess iron in his diet. Although this case study represents only one individual in one institution over the course of one year, IOD has become a major concern for rhinoceroses under human care, and, given the findings, we recommend that institutions with iron-sensitive rhinoceroses sample any and all accessible vegetation as a potential source of iron ingestion.

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Literature Cited

Biemond P, Van Eijk HG, Swaak AJ, & Koster JF (1984) Iron mobilization from ferritin by superoxide derived from stimulated polymorphonuclear leukocytes. Possible mechanism in inflammation diseases. J Clin Invest 73(6): 1576.

Briat JF, Cellier F, and Gaymard F (2006) Ferritins and iron accumulation in plant tissues. In: Barton LL and Abadia J, Eds. Iron Nutrition in Plants and Rhizospheric Microorganisms. pp. 341-357.

Candra D, Radcliffe RW, Khan M, Tsu IH, and Paglia DE (2012) Browse diversity and iron loading in captive Sumatran rhinoceroses (Dicerorhinus Sumatrensis): A Comparison of sanctuary and zoological populations. J Zoo Wildl Med 43(3s): S66-S73.

Clauss M and Hatt JM (2006) The feeding of rhinoceros in captivity. Int Zoo Yearb 40(1): 197-209.

Clauss M, Castell JC, Kienzle E, Schramel P, Dierenfeld ES, Flach EJ, and Hatt JM (2007a) Mineral absorption in the black rhinoceros (Diceros bicornis) as compared with the domestic horse. J Anim Physiol Anim Nutr 91(5-6): 193-204.

Clauss M, Castell JC, Kienzle E, Dierenfeld ES, Flach EJ, Behlert O, and Hatt JM (2007b) The influence of dietary tannin supplementation on digestive performance in captive black rhinoceros (Diceros bicornis). J Anim Physiol Anim Nutr 91(11-12): 449-458.

Clauss M, Dierenfeld E, Goff J, Klasing K, Koutsos L, Lavin S, and Valdes E (2012) IOD in rhinos—nutrition group report: report from the nutrition working group of the international workshop on iron overload disorder in browsing rhinoceros (February 2011). J Zoo Wildl Med 43(3s): S108-S113.

Clauss M and Paglia DE (2012) Iron storage disorders in captive wild mammals: the comparative evidence. J Zoo Wildl Med 43(3s): S6-S18.

de Araújo TO, de Freitas-Silva L, Santana BVN, Kuki KN, Pereira EG, Azevedo AA, and da Silva LC (2014) Tolerance to iron accumulation and its effects on mineral composition and growth of two grass species. Environ Sci Pollut Res Int 21(4): 2777-2784.

Dennis PM, Funk JA, Rajala-Schultz PJ, Blumer ES, Miller RE, Wittum TE, and Saville WJ (2007) A review of some of the health issues of captive black rhinoceroses (Diceros bicornis). J Zoo Wildl Med 38(4): 509-517.

Dobermann A (2000) Rice: nutrient disorders & nutrient management. Volume 1. International Rice Research Institute.

Franco A, Rufo L, Rodríguez N, Amils R, and de la Fuente V (2013) Iron absorption localization and biomineralization of Cynodon dactylon, a perennial grass from the Río Tinto basin (SW Iberian Peninsula). J Plant Nutr Soil Sci 176(6): 836-842.

Helary SF, Shaw JA, Brown D, Clauss M, and Owen-Smith N (2012) Black rhinoceros (Diceros bicornis) natural diets: comparing iron levels across seasons and geographical locations. J Zoo Wildl Med 43(3s): S48-S54.

Horn BE and Area BHM. Mineral Content of Range Grass. University of Wyoming Cooperative Extension Service. https://www.uwyo.edu/uwe/county/johnson/files/mineral-report.pdf. Accessed June 15, 2017.

Kalantar-Zadeh K, Rodriguez RA, and Humphreys MH (2004) Association between serum ferritin and measures of inflammation, nutrition and iron in haemodialysis patients. Nephrol Dial Transplant 19(1): 141-149.

Khan FA, Fisher MA, and Khakoo RA (2007) Association of hemochromatosis with infectious diseases: expanding spectrum. Int J Infect Dis 11(6): 482-487.

Koutsos L, Clauss M, and Valdes E (2016) Designing iron controlled diets for exotic hoofstock – variability in raw materials and manufacturing contributions to total dietary iron. *Proceedings of the Eleventh Symposia of the Comparative Nutrition Society,* Comparative Nutrition Society. Rio Grande, Puerto Rico. pp. 77-80.

Landschoot P (2007) The cool season turfgrasses: basic structures, growth and development. Penn State Cooperative Extension. http://cropsoil.psu.edu/turf/extension/factsheets/cool-season. Accessed June 15, 2017.

Liu H, Zhang J, Christie P, and Zhang F (2008) Influence of iron plaque on uptake and accumulation of Cd by rice (Oryza sativa L) seedlings grown in soil. Sci Total Environ 394(2): 361-368.

Mayland HF and Wilkinson SR (1996) Mineral Nutrition. In: Moser LE, Buxton DR, and Casler MD, Eds. Cool-Season Forage Grasses. pp. 165-191

Miller M, Chavey PS, Hofmeyr J, Mathebula N, Doering A, Buss P, and Olea-Popelka F (2016) Evaluation of serum ferritin and serum iron in free-ranging black rhinoceros (diceros bicornis) as a tool to understand factors affecting iron-overload disorder. J Zoo Wildl Med 47(3): 820-826.

Molenaar FM, Sainsbury AW, Waters M, and Amin R (2008) High serum concentrations of iron, transferrin saturation and gamma glutamyl transferase in captive black rhinoceroses (Diceros bicornis). Vet Rec 162: 716-721.

Paglia DE and Dennis P (1999) Role of chronic iron overload in multiple disorders of captive black rhinoceroses (Diceros bicornis). *Proceedings of the American Association of Zoo Veterinarians Annual Meeting*, American Association of Zoo Veterinarians. 163-171.

Proc. Am. Assoc. Zoo Vet. Annu. Meet (Vol. 1999, pp. 163-171).

Paglia DE and Radcliffe RW (2000) Anthracycline Cardiotoxicity in a Black Rhinoceros (Diceros bicornis): Evidence for Impaired Antioxidant Capacity Compounded by Iron Overload. Vet Pathol 37(1): 86-88.

Paglia DE and Tsu IH (2012) Review of laboratory and necropsy evidence for iron storage disease acquired by browser rhinoceroses. J Zoo Wildl Med 43(3s): S92-S104.

Pessier AP, Munson L, & Miller RE (2004) Oral, nasal, and cutaneous eosinophilic granulomas in the black rhinoceros (Diceros bicornis): a lesion distinct from superficial necrolytic dermatitis. J Zoo Wildl Med 35(1): 1-7.

Pettit RE (2004) Organic matter, humus, humate, humic acid, fulvic acid and humin: Their importance in soil fertility and plant health. http://www.humates.com/pdf/ORGANICMATTERPettit.pdf. Accessed June 16, 2017.

Ruetten M, Steinmetz HW, Clauss M, and Pospischil A (2009) Haemochromatosis in the Black Rhinoceros (Diceros Bicornis Michaeli): Aquired or Congenital?. J Comp Pathol 141(4): 306.

St. John RA, Christians NE, Liu H, Menchyk NA (2013) Secondary Nutrients and Micronutrient Fertilization. In: Stier JC, Horgan BP, and Bonos SA, Eds. Turfgrass: Biology Use and Management. pp. 521-541.

Sullivan KE, Valdes EV, Livingston SE, Knutson MD, Staples CR, Warren LK, and Lavin SR (2015) Use of a novel iron chelator (HBED) in black rhinoceros. In: Bissel H and Brooks M, Eds. *Proceedings of the Eleventh Conference on Zoo and Wildlife Nutrition*, AZA Nutrition Advisory Group. Portland, Oregon. pp. 58-59.

Sullivan K, Livingston S, Williams S, Mylniczenko N, Rodriguez C, Pye G, and Valdes E (2016) Iron Overload Disorder in Browsing Rhinos 2016 Workshop: A review of current goals and practical action planning. *Eleventh Biennial Symposium,* Comparative Nutrition Society. Rio Grande, Puerto Rico.

Terry N and Abadía J (1986) Function of iron in chloroplasts. J Plant Nutr 9(3-7): 609-646.

Varanini Z and Pinton R (2006) Plant-soil relationship: role of humic substances in iron nutrition. In: Barton LL and Abadia J, Eds. Iron Nutrition in Plants and Rhizospheric Microorganisms. pp. 153-168.

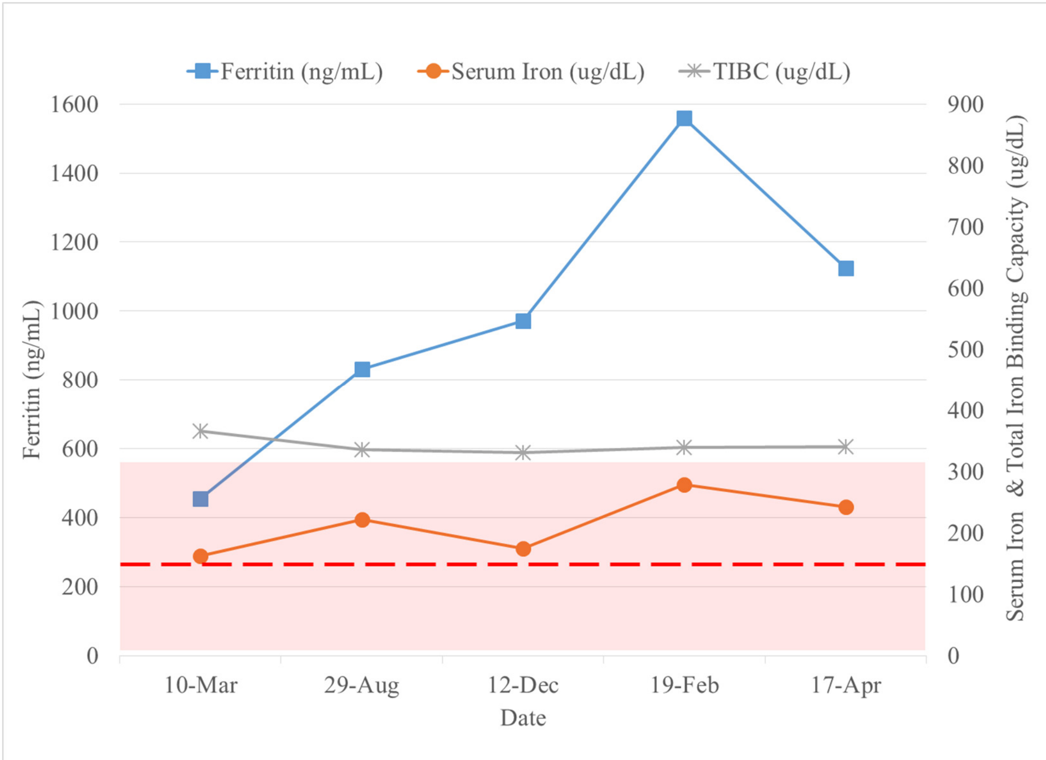

Figure 1. Ferritin, serum iron, and total iron binding capacity over time in a male black rhinoceros housed at Denver Zoo. Red, dashed line depicts wild black rhino adult Ferritin average ± SEM (shaded box); Ferritin = 290.54 ± 247.4 (Miller et al. 2016)

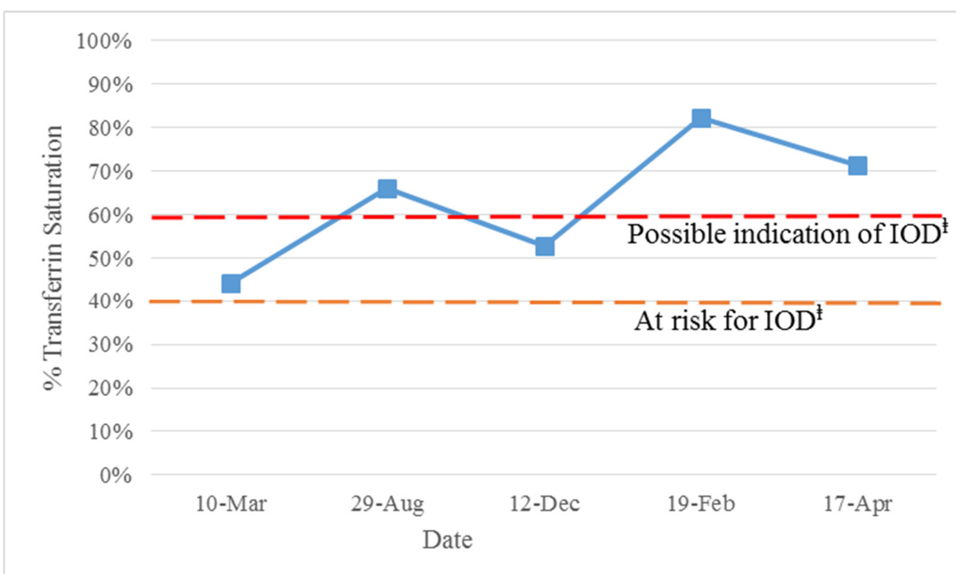

Figure 2. Transferrin saturation over time in a male black rhinoceros housed at Denver Zoo. [ǂ](Molenaar, 2008; Paglia & Dennis, 1999)

Table 1. Prescribed diets fed to a male black rhinoceros at Denver Zoo.

	% of diet, as fed		
Ingredient	**Mar-Oct 2016**	**Oct 2016-present**	**Iron Content (ppm)**
Alfalfa Hay	25.08	0.00	142.00[1]
Timothy Hay	25.08	43.75	106.79[1]
Browser Rhino Diet Cube (5Z1P), Mazuri	11.34	17.32	250.00[2]
Primate Browse Biscuit (5MA4), Mazuri	0.19	0.21	433.32[2]
Alfalfa Cubes, Manzanola Feeds	4.82	4.20	403.31[2]
Bananas, Raw	0.85	0.74	10.36[3]
Carrots, Raw	0.61	0.53	25.62[3]
Apples, Raw, w/Skin	0.71	0.62	8.31[3]
Romaine Lettuce	6.74	5.88	179.96[3]
Ficus spp.	24.46	26.67	151.75[1]
Emcelle	0.02	0.02	0.00[2]
Biotin	0.03	0.00	0.00[2]
Phospha Neutral 250 mg	0.07	0.06	0.00[2]

[1] Sampled on site; analyzed at Dairy One, Inc. Forage Testing Laboratory, Ithaca, New York

[2] From manufacturer information

[3] USDA Food Composition Database, Release 28, 2015

Table 2. Average iron content of pasture and other vegetation throughout Denver Zoo

Feed Item	**Average Iron Content (ppm)**	**n**	**Sampling Period**	**Enclosure**
Grass - Upper Yard	736	2	November 2016	Rhinoceros
Grass - Lower Yard	1,385	2	November 2016	Rhinoceros
Leaves-*Populus* (cottonwood)	497	1	November 2016	Rhinoceros
Leaves-*Fraxinus* (ash)	791	1	November 2016	Rhinoceros
Leaves-*Ulmus* (elm)	973	1	November 2016	Rhinoceros
Leaves-*Celtis* (nettle tree)	622	1	November 2016	Rhinoceros
Seed pods (species unknown)	147	1	November 2016	Rhinoceros
Soil	8.33	3	November 2016	Rhinoceros
Grass - Upper Yard	2110	1	June 2017	Rhinoceros
Grass - Lower Yard	3020	1	June 2017	Rhinoceros
Grass	2855	2	June 2017	Bongo
Grass	265	2	June 2017	Gerenuk
Grass	243	2	June 2017	Kangaroo
Grass	261	2	June 2017	Kudu
Grass	192	2	June 2017	Okapi East
Grass	446	2	June 2017	Okapi West