Inflammatory and oxidative status in European captive black rhinoceroses: a link with Iron Overload Disorder?

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Abstract

Iron Overload Disorder (IOD) is a syndrome developed by captive browsing rhinoceroses like black rhinoceroses (*Diceros bicornis*) in which hemosiderosis settles in vital organs while free iron accumulates in the body, potentially predisposing to various secondary diseases. Captive grazing species like white rhinoceroses (*Ceratotherium simum*) do not seem to be affected. The pro-oxidant and pro-inflammatory properties of iron, associated with the poor antioxidant capacities of black rhinoceroses, could enhance high levels of inflammation and oxidative stress leading to rapid ageing and promoting diseases. In this prospective study, 15 black (BR) and 29 white rhinoceroses (WR) originating from 22 European zoos were blood-sampled and compared for their iron status (serum iron), liver/muscle biochemical parameters (AST, GGT, cholesterol), inflammatory status (total proteins, protein electrophoresis) and oxidative stress markers (SOD, GPX, dROMs). Results showed higher serum iron and liver enzyme levels in black rhinoceroses ($P<0.01$), as well as higher GPX ($P<0.05$) and dROM ($P<0.01$) levels. The albumin/globulin ratio was lower in black rhinoceroses ($P<0.05$) due to higher α_2-globulin levels ($P<0.001$). The present study suggests a higher inflammatory and oxidative profile in captive BR than in WR, possibly in relation to iron status. This could be either a consequence or a cause of iron accumulation, potentially explaining rapid ageing and various diseases. Further investigations are needed to assess the prognostic value of the inflammatory and oxidative markers in captive black rhinoceroses, particularly for evaluating the impact of reduced-iron and antioxidant-supplemented diets.

Introduction

Black rhinoceroses *(Diceros bicornis,* BR) are browser rhinoceroses found in eastern and southern Africa. The three extant wild subspecies, *i.e.* south-western BR (*D. b.* ssp. *bicornis*), eastern BR (*D. b.* ssp. *michaeli*) and southern-central BR (*D. b.* ssp. *minor*), are considered vulnerable to critically endangered by the International Union for Conservation of Nature (IUCN) [1]. Recently, international collaboration enabled the translocation of five BR from three European zoos to Akagera National Park in Rwanda, to diversify the gene-pool and enable healthy population growth in the park [2].

Still, *ex situ* conservation of BR in zoological institutions remains challenging because captive individuals develop several diseases not described in wild BR [3], including hemolytic anemia, hepatopathy, ulcerative dermatopathy and Iron Overload Disorder (IOD). The latter is a syndrome that is being exponentially described in captive BR [4–7], but is not reported in wild BR [4,8–10] nor in grazer rhinoceroses such as white rhinoceroses (*Ceratotherium simum*, WR), whether they be captive or wild. This syndrome is a form of iron storage disease due to free iron accumulation within the organism, leading to hemosiderosis and subsequent hemochromatosis in vital organs, potentially enhancing organ failure in BR [10,11]. The longer the time spent in captivity, the more severe the disease [12]. Currently, the main hypothesis to explain captive BR's susceptibility to iron accumulation is a discrepancy between the captive and the natural diet, which may lead to increased availability of iron in the captive diet [6,11,13–15].

In humans, hemochromatosis is considered as an inflammatory disease [16] with increased oxidative stress [17]. Oxidative stress has severe consequences on health through high tissue and cellular toxicity [16–21] thus participating in cancer formation [18,22] and promoting secondary diseases and rapid ageing. Even finely regulated in the healthy state, non-transferrin bound iron (NTBI, also called free iron) is able to accept and donate electrons readily

thus enhancing the formation of free radicals and consequently oxidative stress [17,23]. Under pathological conditions, iron and superoxide metabolisms are strongly interactive and can exacerbate the toxicity of the other, leading to a self-sustained and ever-increasing spiral of cytotoxic and mutagenic events [17]. This interaction has already been suggested in a BR since they seem to experience a high susceptibility to oxidative stress compared to other mammals [24–29] due to their impaired antioxidant capacities that appeared to be compounded by iron overload.

In the present study, the authors hypothesized that inflammation and oxidative stress may be implicated in the pathogenesis of IOD in captive BR, making this syndrome a potential common denominator to various diseases described in captivity in this species. This study was thus designed to compare inflammation status and oxidative stress levels in relation to iron status in captive BR and WR, the latter being a species theoretically unaffected by IOD.

Materials and methods

Study population

Fifteen BR from 8 European zoos (nine females and six males, aged 5-33 yr-old) and 29 WR from 14 European zoos (18 females and six males, five unknown, aged 4-46 yr-old) were prospectively included between May 2017 and May 2018. Within the 4/15 BR for which the medical background was available, one was reported with infertility, one with ulcerative dermatitis, and one with joint pain or arthritis, whereas the last one had not experienced any infection or illness to the veterinarian's knowledge. Regarding the 21/29 WR for which the medical background was available, 3/21 were reported with arthritis/joint pain, 1/21 with carpal tumour, 1/21 with allergic conjunctivitis and rhinitis, 4/21 with suspected infertility, and the 13/21 remaining had not experienced any infection or illness to the veterinarians' knowledge. None of these captive BR and WR was reported to receive iron chelators.

Sample collection, processing and analysis

This prospective study was validated and supported upstream by coordinators of both BR and WR European Association of Zoos and Aquaria's European Endangered Species Programmes. Each rhinoceros was blood sampled opportunistically by zoo veterinarians, whether during an anaesthesia procedure or a medical training session that was planned to occur independently from the present study. Three millilitre blood samples were collected whether from the auricular or radial vein, both in a heparin and a dry tube. The dry tube was settled for two hours, then centrifuged (1.733 g for five minutes) and the serum was transferred into a clean dry tube. Within 7 days after sampling, both heparinized whole blood and serum tubes were sent to the veterinary laboratory (LDHVet-LabOniris, Nantes, France). Median delay between the sampling and the reception by the lab was 3 days [range from 1 to 7 days] including 2 days of shipment [range from 1 to 3 days]. Directly after reception, whole blood and serum were aliquoted and stored at -20°C until analyses were performed.

All the serum biochemistry was performed using an automated biochemistry analyser (RX Daytona, Randox Laboratories, Crumlin, County Antrim, United Kingdom), unless indicated otherwise. Iron status was evaluated through serum iron measurement (ferrozine colorimetric method). As the liver and muscle are reported as the first tissues suffering from IOD in BR [30], the hepatic and muscular functions were investigated through the measurement of the following parameters: aspartate aminotransferase (AST, L-aspartate/α-oxoglutarate as substrate), gamma glutamyltransferase (GGT, L-γ-glutamyl-3-carboxy-4-nitroanilide/glycylglycine as substrate), cholesterol (cholesterol esterase/oxidase method) and creatine kinase (CK, creatine phosphate as substrate). Inflammation status was evaluated by measuring total serum protein (TP, biuret method) and agarose gel electrophoretic albumin and globulin fractions (albumin and globulins including α_1-, α_2-, β- and γ-globulin fractions,

agarose gel method (Hyrys2, Hydragel; Sebia, Evry, France). Globulin fractions were determined according to recent published data from Hooijberg and colleagues [31]. α_1-globulins refers to α_1-a and α_1-b fractions, and β-globulins refers to β1 and β2 fractions. The albumin:globulin ratio (A/G) was calculated. Finally, oxidative stress was assessed through the measurement of superoxide dismutase (SOD) and glutathione peroxidase (GPX) activities (colorimetric methods with RANSOD and RANSEL test kits respectively), and reactive oxygen metabolites (dROMs, Diacron Reactive Oxygen Metabolites d-ROMs test, Diacron Laboratories Grosseto, Italy).

No heparinized whole blood was received for one of the BR in which GPX and SOD could thus not be measured. A blood clot was observed in the heparin tube of one WR. As a consequence, GPX, SOD and dROMs could not be measured. CK levels were measured after all the other analyses: as such, this information is missing for two BR and three WR for which serum was lacking. Finally, in one of the WR, protein electrophoresis showed aberrant results which remained unexplained and it was thus excluded from the results.

Statistical analyses

All statistical analyses were carried out using R-Studio software version 1.1.442 [32]. The data were pooled across species, and basic descriptive statistics, including arithmetic mean, median, standard deviation (SD), minimum and maximum, were obtained for each parameter. As no variable was normally distributed, non-parametric statistical tests were used. Mann Whitney test was performed for all the quantitative parameters (age, iron, AST and CK activities, cholesterol, total proteins, albumin, SOD and GPx) in order to compare BR and WR. Graphic assessment of the SOD and GPx data showed a potential discriminant threshold (>1500 g/Hb and >300 U/gHb, respectively) between the 2 populations of rhinos. As a result, these data were transformed into categorical variables (“Normal” versus “High”) for the statistical

136 analysis. For GGT and dROMs values, as some results were out of the linearity range of the
137 analyser (10 rhinos had GGT values <8 U/L and 13 had dROMs > 1,000 UCARR), a 2 level
138 categorization was also performed using a graphically determined threshold (13 U/L for GGT
139 and 800 UCARR for dROMs). Chi-squared tests were used to compare BR and WR for sex,
140 CatGGT, CatdROMs, CatSOD and CatGPX. Statistical significance was set at $P < 0.05$.
141

142 # Results

143 Measured parameters for both European captive BR and WR are listed in Table 1. Sex
144 distribution did not differ significantly between BR and WR (P=0.32) as well as age (median
145 21 years [5-33] versus 16 years [4-46], respectively) (P = 0.32). Serum iron was significantly
146 higher ($P < 0.01$) in BR (median 42.0 [26.6-58.9] µmol/L) compared to WR (28.0 [11.1-58.4]
147 µmol/L) (Fig 1). Regarding liver and muscular function, AST activity (96 [72-152] versus 71
148 [12-178] U/L for BR and WR, respectively) (Fig 1), CatGGT (100% "High" *i.e* GGT>13 U/L
149 versus 20.8% for BR and WR, respectively) and CK (323 [199-945] versus 196 [129-697] U/L
150 for BR and WR, respectively) were significantly higher ($P< 0.01$) in BR compared to WR. Total
151 proteins were significantly higher ($P = 0.03$) in BR (84 [65-92] g/L) compared to WR (78 [70-
152 95] g/L) (Fig 1). A/G ratio was significantly lower ($P = 0.01$) in BR (0.56 [0.31-0.83]) compared
153 to WR (0.73 [0.20-1.07]) because of significantly higher ($P < 0.001$) levels of α_2-globulin (16.2
154 [10.2-21.9] g/L and 11.2 [6.7-15.8] g/L for BR and WR, respectively) (Figs 1 and 2). Finally,
155 regarding oxidative stress assessment, CatdROMs was significantly higher ($P < 0.001$) in BR
156 (93.3 % "High" *i.e* dROMs > 800 UCARR) in comparison to WR (32.1% "High" *i.e* dROMs
157 > 800 UCARR), as well as CatGPX ($P = 0.047$; 64.3% versus 32.1% of "High" *i.e.* GPX > 300
158 U/gHb, for BR and WR, respectively).
159

160 **Fig 1. Boxplots showing the quartiles and outliers of serum iron, AST, TP and α_2-**

globulins results in 15 European captive black rhinoceroses (BR) and 29 white rhinoceroses (WR). Values were significantly different between the species in all cases ($P < 0.05$).

Fig 2. Serum protein electrophoreses showing an example of the serum protein distribution in a black rhinoceros (BR, total proteins 92 g/L, α_2 globulins 27 g/L) and a white rhinoceros (WR, total proteins 75 g/L, α_2 globulins 9.6 g/L). Note the increased size of the α_2 globulin region in the BR compared to the WR (grey areas).

Table 1. Results for parameters measured in European captive black and white rhinoceroses for the evaluation of iron status, hepatic and muscular function, inflammation status and oxidative stress levels.[a]

	Black rhinoceros							White rhinoceros						
	Mean	**SD**	**Min**	**Median**	**Max**	**"High"**	**n**	**Mean**	**SD**	**Min**	**Median**	**Max**	**"High"**	**n**
Age (years)	19	9.2	5	21	33		15	16	10.0	4	16	46		26
Serum iron (µmol/L)	42.2	10.7	26.6	42.0[b]	58.9		15	29.8	9.9	11.1	28.0	58.4		29
AST (U/L)	104	21.4	72	96[b]	152		15	77	33.4	12	71	178		29
CK (U/L)	379	201.7	199	323[b]	945		13	246	142.9	129	196	697		26
GGT (U/L)	25	8.1	14	24	45		15	c	c	< 8	10	20		29
CatGGT (%)						100[b]	15						20.8	29
Cholesterol (g/L)	0.7	0.2	0.5	0.7	1.3		15	0.8	0.4	0.4	0.7	2.6		29
TP (g/L)	82.0	8.3	65.0	84.0[b]	92.0		15	78.2	5.7	70.0	78.0	95.0		29
A/G ratio	0.57	0.16	0.31	0.56[b]	0.83		15	0.71	0.17	0.20	0.73	1.07		28
Albumin (g/L)	29.4	6.6	15.3	31.5	38.0		15	32.1	5.9	13.3	32.4	40.4		28
α_1-globulin (g/L)	6.2	1.2	4.0	6.3	7.8		15	6.1	0.8	4.6	6.1	7.6		28
α_2-globulin (g/L)	15.8	3.0	10.2	16.2[b]	21.9		15	11.3	1.9	6.7	11.2	15.8		28
ß-globulin (g/L)	16.4	3.4	11.9	17.1	21.8		15	16.5	2.8	13.4	15.9	26.1		28
γ-globulin (g/L)	14.0	4.2	8.1	12.9	23.7		15	12.4	2.9	7.7	12.4	22.5		28
SOD (U/g Hb)	1737	340.3	1130	1730	2400		14	1636	481.3	900	1505	3300		28
CatSOD (%)						50							71.4	
GPX (U/g Hb)	324	134.2	104	346	560		14	240	113.8	47	239	423		28
CatGPX (%)						64.3[b]							32.1	
dROMs (U CARR)	c	c	416	978	> 1,000		15	c	c	380	686	> 1,000		28
CatdROMs (%)						93.3[b]	15						32.1	28

[a] A/G ratio indicates albumin/globulin ratio; CatGGT, GGT values categorized as "Normal" or "High" (> 13 U/L); CatSOD, SOD values categorized as "Normal" or "High" (> 1,500 U/gHb); CatGPX, GPX values categorized as "Normal" or "High" (> 300 U/g/Hb); CatdROMs, dROMs categorized as "Normal" or "High" (> 800 UCARR).

[b] Statistically significant ($P < 0.05$) differences between black rhinoceroses and white rhinoceroses.

c Not calculated because of values out of measurable assay range.

Discussion

This study showed that European captive black rhinoceroses exhibited higher serum iron concentration and higher inflammatory and oxidative status than captive white rhinoceroses. Taken together, these findings suggest that BR could be predisposed to iron accumulation probably leading to IOD and enhancing inflammatory and oxidative states.

Serum iron values found for the European captive BR included in the present study were very similar to those available in the literature regarding European [10] and American [14,33] captive BR. Serum iron levels being higher in captive BR compared to captive WR has already been reported [8,33]. As such, these results could confirm a predisposition of captive European BR to develop IOD, as previously described [34]. Even if liver biopsy remains the gold standard for definitive diagnosis of iron overload syndromes [35] and has already been performed in a live captive BR confirming diffuse hemosiderosis [36], this procedure is technically challenging due to the animal's size, the depth of the liver, the difficulty of ultrasound and the skin thickness. Despite having been used in several studies [8,37], ferritin is not specific for iron overload syndromes [38] and is reported as a poor biomarker for IOD progression in Sumatran rhinoceroses, another species of browser rhinoceros [39]. As a consequence, serum iron, TIBC (Total Iron Binding Capacity) measurement and subsequent calculation of Transferrin Saturation may be currently the best tools for guiding *ante-mortem* diagnosis and prognosis of IOD in captive BR as suggested by several studies [10,33,34,36] and should be included in regular blood tests when checking for the health status of captive BR. Measurement of the Total Iron Binding Capacity (TIBC) was intended in the present study through a direct method (TIBC_2, colorimetric method, RX Daytona, Randox Laboratories, Crumlin, County Antrim, United Kingdom), but results were aberrant with many values of TIBC greater than the serum iron concentration, leading to a calculated Transferrin Saturation greater than 100%, which is physiologically not possible. Briefly, in this method, iron is first removed from

transferrin through acidification of the sample and a known amount of iron is added. All iron is then complexed and coloured with an iron-binding dye, and absorbance is measured. A second reagent is then added, making the pH rise, resulting in a large increased affinity of transferrin for iron. The observed decrease in absorbance of the coloured dye-iron complex is directly proportional to the TIBC of the serum [40]. We hypothesized that transferrin from rhinoceroses was not as pH-sensitive as human transferrin, and that pH variations were not adequate to accurately measure TIBC in this species, leading to underestimated values. Other methods, such as evaluation of unbound iron binding capacity (UIBC) might be more suitable to calculate Transferrin Saturation in rhinoceroses, as reported in humans [41,42].

Liver function was assessed through AST and GGT measurements, as recommended for domestic horses [43], especially when investigating hemochromatosis [44,45]: indeed, the horse is considered as a very good domestic model animal for rhinoceroses [46,47]. In the present study, increased values of AST in BR compared to WR could have two main causes, including compromised liver function as suggested by increased GGT, and muscular lesions as suggested by increased CK. Molenaar and colleagues reported similar results for GGT in European captive BR [10]. IOD could be implicated in both hepatic and muscular dysfunctions. Indeed, the liver is the first site of hemochromatosis when IOD develops in BR, which impairs its function [16,30]. Iron deposition in muscles has been reported in BR affected by IOD [30]. As hemochromatosis, the latest stage of hemosiderosis, is a progressive and irreversible process with fibrosis that eventually leads to hepatic cirrhosis or carcinoma and fatality in humans [48–50], hepatic biochemistry parameter measurements could be useful prognostic factors in BR affected by IOD.

Higher TP and decreased A/G ratio due to an increase of the α_2-globulin fraction were observed in captive BR compared to WR, highly suggestive of an increased inflammatory state [51], as it is described in humans affected by hemochromatosis [16,52]. In this study, selecting

captive WR instead of free-ranging BR as the negative control precisely aimed at limiting the captivity bias since captivity may be pro-inflammatory by itself [37,53]. Serum protein electrophoretic results of the captive WR included in this study showed some variations in comparison to healthy free-ranging WR [31], further underlining the interest of selecting captive WR as a control group in order to limit the captivity bias. Obesity is described for being pro-oxidative and pro-inflammatory [54], but no study to date investigates the consequences of overweight in rhinoceroses. It would have been relevant to determine whether the European captive BR population did not exhibit significant obesity compared to the European captive WR at the time of the study. However, even if a body condition scoring system has been proposed for BR and Indian rhinoceroses [55,56], no such system exists for WR. The main proteins migrating in the α_2 region on serum protein electrophoresis include haptoglobin, α_2-macroglobulin, ceruloplasmin and serum amyloid A (SAA). Since Smith and colleagues have already reported that haptoglobin levels were not significantly different between 10 BR and 20 WR kept in captivity [8], it may not play an important role in the BR's α_2-globulin increase in the present study. α_2-macroglobulin inhibits numerous endogenous proteases and acts as a transport protein for cytokines and growth factors [57]. Increased α_2-macroglobulin is favored by inflammatory states, such as diabetes mellitus in humans [57]. Ceruloplasmin levels increase with inflammation in humans [58]. Among many roles, this ferroxidase helps to reduce circulating free ferrous iron [17]. As a consequence, it could be hypothesized that ceruloplasmin levels may increase in BR with iron accumulation in response to inflammation and high levels of circulating free iron. As such, its measurement could be of interest in future studies on IOD in BR. Finally, SAA, that increases during inflammation, was reported to be significantly higher in captive BR compared to wild BR [53]. As a result, an increase in α_2-macroglobulin, ceruloplasmin and SAA, which all suggest inflammation, could explain the increase in α_2-globulin observed in the captive BR in this study. This finding suggests that IOD could be a

chronic inflammatory disease like hemochromatosis is in humans [16]. Inflammation is known for inducing tissue iron storage through hepcidin stimulation and as a consequence for progressively leading to hemochromatosis, thus aggravating iron overload syndromes [59]. Inflammation could thus be a cause and a consequence of IOD in BR, making IOD a potential self-sustaining disease if no iron overload or inflammatory state management is undertaken, with regular phlebotomies as previously described [60].

dROMs are hydroperoxydes, meaning reactive oxygen metabolites that allow to directly evaluate oxidative stress levels [61,62]. SOD and GPX are antioxidant enzymes whose activity measurement indirectly allows to quantifying the response to oxidative stress [63,64]. In the present study, significantly higher levels of GPX and dROMs were observed in captive BR compared to WR, suggesting a higher degree of oxidative stress in captive BR. Increase in oxidative stress levels in captive BR could be due to their lower antioxidant capacities [25,28,29], captivity itself [37,53] and/or a self-sustaining process in which oxidative stress disrupts antioxidant defences [63]. High levels of oxidative stress can predispose to diseases and rapid ageing [17] and should be taken into consideration for husbandry, health care and more globally *ex-situ* conservation of endangered species like the BR. Oxidative stress is reported to worsen iron overload syndromes like iron overload cardiomyopathies in humans [65], which themselves favour reactive oxygen metabolite formation and thus oxidative stress increase [17]. IOD could then be self-sustained through the oxidative stress induced, leading to a vicious circle. However, no significant difference was found concerning SOD. One explanation may be that SOD is not as implicated in BR's antioxidant defences as in other mammals, making this biomarker possibly not adapted to this species. Regardless, due to low animal numbers, these results should be interpreted with caution.

The main limit of the present study is the postulate that all captive BR may be affected by IOD whereas captive WR are not, without performing a liver biopsy for a definitive diagnosis of

IOD. As such, the hypothesized link between increased inflammation and oxidative stress levels and the development of IOD in captive BR need further evaluations to be confirmed. Pre-analytical homogeneity is sub-optimal in the present study. Indeed, food, husbandry and medical management including treatments administered to captive BR and WR could not be controlled. Even if none of their diet included iron chelators, the amount of iron in each diet was not assessed. Also, each zoo institution that collected the blood samples performed the first steps of the processing *i.e.* centrifugation and serum transfer to a new dry tube. The detailed collection and processing protocol submitted to the institutions aimed at limiting this bias. After reception, all samples were aliquoted, frozen and stored at the laboratory for varying durations before they were analysed: this was done in order to group the tests. In humans, most common biochemical analytes show adequate stability in serum following 30 days of storage at −20°C [66], which was the temperature used in the present study for aliquot storage. Nevertheless, it cannot be ruled out that those pre-analytical variations may have compromised the reliability of some blood test results, as no study is available on the stability of such parameters in rhinoceros blood samples.

Conclusions

Findings of the present study suggest that captive Black Rhinoceroses exhibit higher iron concentrations, higher inflammatory status and higher oxidative stress levels than captive White Rhinoceroses. Taken together, these findings suggest that BR could be predisposed to iron accumulation probably leading to IOD and enhancing inflammatory and oxidative states. Both oxidative stress and inflammation may favour secondary diseases, rapid ageing, and aggravation of IOD, tissue hemochromatosis and organ fibrosis. Thus, efforts to control IOD progression in this endangered species when kept in captivity should be continued, whether through iron level control in the diet or through regular therapeutic phlebotomies. Further

investigations are needed to assess the prognostic value of the inflammatory and oxidative markers in captive BR, particularly for evaluating the impact of reduced-iron and antioxidant-supplemented diets.

Acknowledgments

The authors would like to thank the 22 European zoos that have participated in this prospective study by sending blood samples of their hosted black and white rhinoceroses, and LDHVet - LabOniris for performing the blood analyses.

References

1. Emslie R. Diceros bicornis. In: The IUCN Red List of Threatened Species 2012: e.T6557A16980917 [Internet]. 2012 [cited 21 Aug 2019]. Available: http://dx.doi.org/10.2305/IUCN.UK.2012.RLTS.T6557A16980917.en

2. Pereira E. Five rhinos from European zoos are heading to Rwanda. In: Save The Rhino [Internet]. 21 Jun 2019 [cited 21 Aug 2019]. Available: https://www.savetherhino.org/rhino-species/black-rhino/five-black-rhinos-born-in-european-zoos-are-heading-to-africa/

3. Dennis PM, Funk JA, Rajala-Schultz PJ, Blumer ES, Miller RE, Wittum TE, et al. A review of some of the health issues of captive black rhinoceroses (*Diceros bicornis*). J Zoo Wildl Med. 2007;38: 509–517. doi:10.1638/MS05-012.1

4. Ganz T, Goff J, Klasing K, Nemeth E, Roth T. IOD in rhinos - Immunity group report: report from the immunity, genetics and toxicology working group of the International Workshop on Iron Overload Disorder in Browsing Rhinoceros. J Zoo Wildl Med. 2012;43: S117–S119.

5. Citino S, Bryant B, Duncan M, Fleming G, Hofmeyr M, Miller E, et al. IOD in rhinos - Veterinary group report: report from the clinical medicine and pathology working group of the international workshop on Iron Overload Disorder in browsing rhinoceros. J Zoo Wildl Med. 2012;43: S105–S107.

6. Clauss M, Dierenfeld E, Goff J, Klasing K, Koutsos L, Lavin S, et al. IOD in rhinos - Nutrition group report: report from the nutrition working group of the International Workshop on Iron Overload Disorder in Browsing Rhinoceros. J Zoo Wildl Med. 2012;43: S108–S113.

7. Dennis P, Ellis S, Mellen J, Lee P, Olea-Popelka F, Petric A, et al. IOD in rhinos - Epidemiology group report: report from the epidemiology working group of the

359 International Workshop on Iron Overload Disorder in Browsing Rhinoceros. J Zoo Wildl
360 Med. 2012;43: S114–S116. doi:10.1638/1042-7260-43.3s.S114

361 8. Smith JE, Chavey PS, Miller RE. Iron metabolism in captive black (*Diceros bicornis*) and
362 white (*Ceratotherium simum*) rhinoceroses. J Zoo Wildl Med. 1995; 525–531.

363 9. Paglia DE, Tsu I-H. Review of laboratory and necropsy evidence for iron storage disease
364 acquired by browser rhinoceroses. J Zoo Wildl Med. 2012;43: S92–S104.
365 doi:10.1638/2011-0177.1

366 10. Molenaar FM, Sainsbury AW, Waters M, Amin R. High serum concentrations of iron,
367 transferrin saturation and gamma glutamyl transferase in captive black rhinoceroses
368 (*Diceros bicornis*). Vet Rec. 2008;162: 716–721. doi:10.1136/vr.162.22.716

369 11. Paglia DE. Iron storage syndrome in rhinoceroses: potential role for rhino keepers in
370 prevention and therapy. Fourth Rhino Keepers Workshop. Colombus, Ohio; 2005. pp. 1–
371 10.

372 12. Kock N, Foggin C, Kock MD, Kock R. Hemosiderosis in the black rhinoceros (*Diceros*
373 *bicornis*): a comparison of free-ranging and recently captured with translocated and
374 captive animals. J Zoo Wildl Med. 1992; 230–234.

375 13. Wright JB. Essential fatty acids, total lipid, and condensed tannin in the diet of captive
376 black rhinoceroses (*Diceros bicornis*) in North America and in browses native to
377 Zimbabwe, Africa. Cornell University, Thesis presented to the Faculty of the Graduate
378 School. 1998.

379 14. Paglia DE, Dennis P. Role of chronic iron overload in multiple disorders of captive black
380 rhinoceroses (*Diceros bicornis*). Proc AAZV. 1999. pp. 163–171.

381 15. Castell JC. Investigations on feeding and digestive physiology of the black rhinoceros
382 (*Diceros bicornis*). University of Munich. 2005.

383 16. Ramm GA, Ruddell RG. Iron homeostasis, hepatocellular injury, and fibrogenesis in
384 hemochromatosis: the role of inflammation in a noninflammatory liver disease. Semin
385 Liver Dis. 2010;30: 271–287. doi:10.1055/s-0030-1255356

386 17. Emerit J, Beaumont C, Trivin F. Iron metabolism, free radicals, and oxidative injury.
387 Biomed Pharmacother. 2001;55: 333–339. doi:10.1016/S0753-3322(01)00068-3

388 18. Zhuang T, Han H, Yang Z. Iron, oxidative stress and gestational diabetes. Nutrients.
389 2014;6: 3968–3980. doi:10.3390/nu6093968

390 19. Ruivard M. Les chélateurs du fer : quand et comment les utiliser chez l'adulte ? Rev
391 Médecine Interne. 2013;34: 32–38. doi:10.1016/j.revmed.2012.05.005

392 20. McCord JM. Effects of positive iron status at a cellular level. Nutr Rev. 1996;54: 85–88.

393 21. Bhat AH, Dar KB, Anees S, Zargar MA, Masood A, Sofi MA, et al. Oxidative stress,
394 mitochondrial dysfunction and neurodegenerative diseases; a mechanistic insight. Biomed
395 Pharmacother Biomedecine Pharmacother. 2015;74: 101–110.
396 doi:10.1016/j.biopha.2015.07.025

22. Gozzelino R, Arosio P. Iron Homeostasis in Health and Disease. Int J Mol Sci. 2016;17: 130. doi:10.3390/ijms17010130

23. Muñoz M, García-Erce JA, Remacha AF. Disorders of iron metabolism. Part 1: molecular basis of iron homoeostasis. J Clin Pathol. 2011;64: 281–286. doi:10.1136/jcp.2010.079046

24. Chaplin H, Malecek AC, Miller RE, Bell CE, Gray LS, Hunter VL. Acute intravascular hemolytic anemia in the black rhinoceros: hematologic and immunohematologic observations. Am J Vet Res. 1986;47: 1313–1320.

25. Paglia DE, Miller RE. Erythrocytes of the Black rhinoceros Diceros bicornis: susceptibility to oxidant-induced haemolysis. Int Zoo Yearb. 1993;32: 20–27. doi:10.1111/j.1748-1090.1993.tb03510.x

26. Paglia DE. Acute episodic hemolysis in the African black rhinoceros as an analogue of human glucose-6-phosphate dehydrogenase deficiency. Am J Hematol. 1993;42: 36–45.

27. Paglia DE, Miller RE, Renner SW. Is impairment of oxidant neutralization the common denominator among diverse diseases of black rhinoceroses? Proc AAZV. 1996.

28. Paglia DE, Radcliffe RW. Anthracycline cardiotoxicity in a black rhinoceros (*Diceros bicornis*): evidence for impaired antioxidant capacity compounded by Iron Overload. Vet Pathol Online. 2000;37: 86–88. doi:10.1354/vp.37-1-86

29. Harley EH, Matshikiza M, Robson P, Weber B. Red blood cell metabolism shows major anomalies in Rhinocerotidae and Equidae, suggesting a novel role in general antioxidant metabolism. Int Congr Ser. 2004;1275: 334–340. doi:10.1016/j.ics.2004.08.062

30. Olias P, Mundhenk L, Bothe M, Ochs A, Gruber AD, Klopfleisch R. Iron Overload Syndrome in the Black Rhinoceros (*Diceros bicornis*): Microscopical Lesions and Comparison with Other Rhinoceros Species. J Comp Pathol. 2012;147: 542–549. doi:10.1016/j.jcpa.2012.07.005

31. Hooijberg EH, Miller M, Cray C, Buss P, Steenkamp G, Goddard A. Serum protein electrophoresis in healthy and injured southern white rhinoceros (*Ceratotherium simum simum*). PLOS ONE. 2018;13: e0200347. doi:10.1371/journal.pone.0200347

32. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing; 2017. Available: https://www.R-project.org/

33. Dierenfeld ES, Atkinson S, Craig AM, Walker KC, Streich WJ, Clauss M. Mineral concentrations in serum/plasma and liver tissue of captive and free-ranging Rhinoceros species. Zoo Biol. 2005;24: 51–72. doi:10.1002/zoo.20043

34. Clauss M, Paglia DE. Iron storage disorders in captive wild mammals: the comparative evidence. J Zoo Wildl Med. 2012;43: S6–S18. doi:10.1638/2011-0152.1

35. Adams PC, Beaton MD. Transferrin saturation as a predictor of hepatic iron overload. Liver Int Off J Int Assoc Study Liver. 2011;31: 272–273. doi:10.1111/j.1478-3231.2010.02335.x

435 36. Mylniczenko ND, Sullivan KE, Corcoran ME, Fleming GJ, Valdes EV. Management
436 strategies of iron accumulation in a captive population of black rhinoceroses (*Diceros*
437 *bicornis minor*). J Zoo Wildl Med. 2012;43: S83–S91. doi:10.1638/2011-0168.1

438 37. Miller M, Chavey PS, Hofmeyr J, Mathebula N, Doering A, Buss P, et al. Evaluation of
439 serum ferritin and serum iron in free-ranging black rhinoceros (*Diceros bicornis*) as a tool
440 to understand factors affecting Iron Overload Disorder. J Zoo Wildl Med. 2016;47: 820–
441 826. doi:10.1638/2015-0295.1

442 38. Kalantar-Zadeh K, Rodriguez RA, Humphreys MH. Association between serum ferritin
443 and measures of inflammation, nutrition and iron in haemodialysis patients. Nephrol Dial
444 Transplant. 2004;19: 141–149. doi:10.1093/ndt/gfg493

445 39. Roth TL, Reinhart PR, Kroll JL. Serum ferritin concentration is not a reliable biomarker
446 of Iron Overload Disorder progression or hemochromatosis in the Sumatran rhinoceros
447 (*Dicerorhinus sumatrensis*). J Zoo Wildl Med. 2017;48: 645–658. doi:10.1638/2017-
448 0010.1

449 40. Siek G, Lawlor J, Pelczar D, Sane M, Musto J. Direct serum total iron-binding capacity
450 assay suitable for automated analyzers. Clin Chem. 2002;48: 161–166.
451 doi:10.1093/clinchem/48.1.161

452 41. Yamanishi H, Iyama S, Yamaguchi Y, Kanakura Y, Iwatani Y. Total iron-binding
453 capacity calculated from serum transferrin concentration or serum iron concentration and
454 unsaturated iron-binding capacity. Clin Chem. 2003;49: 175–178. doi:10.1373/49.1.175

455 42. Adams PC. Epidemiology and diagnostic testing for hemochromatosis and iron overload.
456 Int J Lab Hematol. 2015;37: 25–30. doi:10.1111/ijlh.12347

457 43. Ambrojo KS, Poggi JCG, Juzado AM. Use of laboratory testing to diagnose liver and
458 biliary dysfunction in the horse. J Gastroenterol Hepatol Res. 2013;2: 807–813.
459 doi:10.6051/j.issn.2224-3992.2013.02.344

460 44. Theelen MJP, Beukers M, Grinwis GCM, Sloet van Oldruitenborgh-Oosterbaan MM.
461 Hemochromatosis and liver failure in 11 horses due to chronic iron intoxication. Proc 7th
462 Congress of the European College of Equine Internal Medicine. Prague, Czech Republic;
463 2014. p. 15.

464 45. Olsman AFS, Sloet van Oldruitenborgh-Oosterbaan MM. Primary liver disease in the
465 horse. Tijdschr Diergeneeskd. 2004; 510–522.

466 46. Nielsen BD, Vick MM, Dennis PM. A potential link between insulin resistance and Iron
467 Overload Disorder in browsing rhinoceroses investigated through the use of an equine
468 model. J Zoo Wildl Med. 2012;43: S61–S65. doi:10.1638/2011-0145.1

469 47. Clauss M, Castell JC, Kienzle E, Schramel P, Dierenfeld ES, Flach EJ, et al. Mineral
470 absorption in the black rhinoceros (*Diceros bicornis*) as compared with the domestic
471 horse. J Anim Physiol Anim Nutr. 2007;91: 193–204. doi:10.1111/j.1439-
472 0396.2007.00692.x

48. Miller M. Effect of venipuncture site and anticoagulant on selected hematologic values in black rhinoceros (*Diceros bicornis*). J Zoo Wildl Med. 2003;34: 59–64. doi:10.1638/1042-7260(2003)34[0059:EOVSAA]2.0.CO;2

49. Aziza SAH, Azab ME-S, El-Shall SK. Ameliorating role of rutin on oxidative stress induced by iron overload in hepatic tissue of rats. Pak J Biol Sci PJBS. 2014;17: 964–977. doi:10.3923/pjbs.2014.964.977

50. Paglia DE. An historical perspective: Iron Overload Disease in browser rhinoceroses. Keeper workshop; 2015; Chester.

51. O'Connell T, Horita TJ, Kasravi B. Understanding and interpreting the serum protein electrophoresis. Am Fam Physician. 2005;71: 105–112.

52. Hübscher SG. Iron overload, inflammation and fibrosis in genetic haemochromatosis. J Hepatol. 2003;38: 521–525. doi:10.1016/s0168-8278(03)00078-3

53. Schook MW, Wildt DE, Raghanti MA, Wolfe BA, Dennis PM. Increased inflammation and decreased insulin sensitivity indicate metabolic disturbances in zoo-managed compared to free-ranging black rhinoceros (*Diceros bicornis*). Gen Comp Endocrinol. 2015;217–218: 10–19. doi:10.1016/j.ygcen.2015.05.003

54. Sfar S, Boussoffara R, Sfar MT, Kerkeni A. Antioxidant enzymes activities in obese Tunisian children. Nutr J. 2013;12: 18. doi:10.1186/1475-2891-12-18

55. Reuter H, Adcock K. Standardised body condition scoring system for black rhinoceros (*Diceros bicornis*). Pachyderm. 1998;26: 116–121.

56. Heidegger EM, Houwald F von, Steck B, Clauss M. Body condition scoring system for greater one-horned rhino (*Rhinoceros unicornis*): Development and application. Zoo Biol. 2016;35: 432–443. doi:10.1002/zoo.21307

57. Takada T, Kodera Y, Matsubara M, Kawashima Y, Maeda T, Fujita Y, et al. Serum monomeric α2-macroglobulin as a clinical biomarker in diabetes. Atherosclerosis. 2013;228: 270–276. doi:10.1016/j.atherosclerosis.2013.02.035

58. Hammadah M, Fan Y, Wu Y, Hazen SL, Wilson Tang WH. Prognostic value of elevated serum ceruloplasmin levels in patients with heart failure. J Card Fail. 2014;20: 946–952. doi:10.1016/j.cardfail.2014.08.001

59. Yanoff L, Menzie C, Denkinger B, Sebring N, McHugh T, Remaley A, et al. Inflammation and iron deficiency in the hypoferremia of obesity. Int J Obes 2005. 2007;31: 1412–1419. doi:10.1038/sj.ijo.0803625

60. Paglia DE. Recommended phlebotomy guidelines for prevention and therapy of captivity-induced iron-storage disease in rhinoceroses, tapirs and other exotic wildlife. Proc AAZV. 2004; 122–127.

61. Colombini F, Carratelli M, Alberti A. Oxidative stress, d-ROMs test, and ceruloplasmin. Free Radic Res. 2016;50: 447–453. doi:10.3109/10715762.2015.1136063

510 62. Ito F, Ito T, Suzuki C, Yahata T, Ikeda K, Hamaoka K. The application of a modified d-
511 ROMs test for measurement of oxidative stress and oxidized high-density lipoprotein. Int
512 J Mol Sci. 2017;18. doi:10.3390/ijms18020454

513 63. Bayraktar N, Kilic S, Bayraktar MR, Aksoy N. Lipid peroxidation and antioxidant enzyme
514 activities in cancerous bladder tissue and their relation with bacterial infection: a
515 controlled clinical study. J Clin Lab Anal. 2010;24: 25–30. doi:10.1002/jcla.20356

516 64. Lucas ML, Carraro CC, Belló-Klein A, Kalil AN, Aerts N. Oxidative stress in human aorta
517 of patients with advanced aortoiliac occlusive disease. Braz J Cardiovasc Surg. 2016;31:
518 428–433. doi:10.5935/1678-9741.20160086

519 65. Cheng C-F, Lian W-S. Prooxidant mechanisms in iron overload cardiomyopathy. BioMed
520 Res Int. 2013;2013: 1–8. doi:10.1155/2013/740573

521 66. Kachhawa K, Kachhawa P, Varma M, Behera R, Agrawal D, Kumar S. Study of the
522 stability of various biochemical analytes in samples stored at different predefined storage
523 conditions at an accredited laboratory of India. J Lab Physicians. 2017;9: 11–15.
524 doi:10.4103/0974-2727.187928

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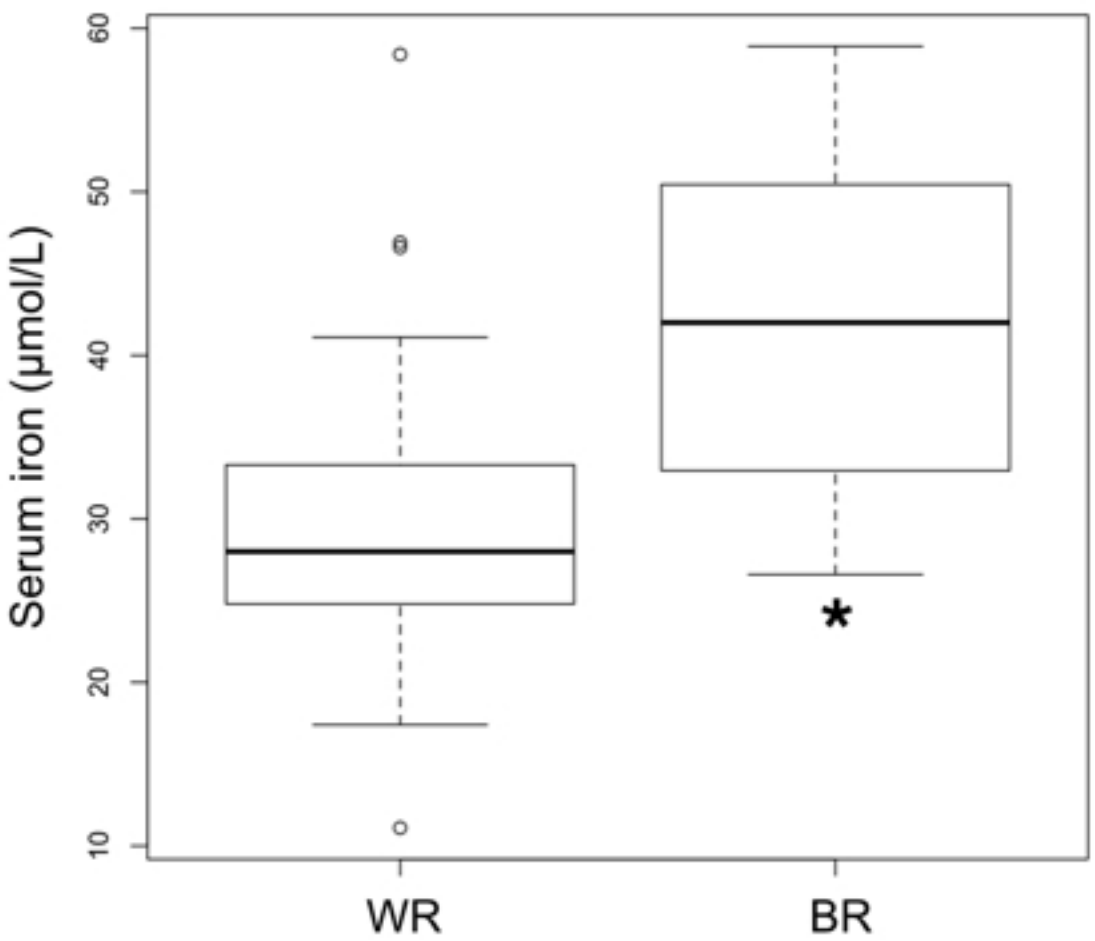
Serum iron (μmol/L)
10
20
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50
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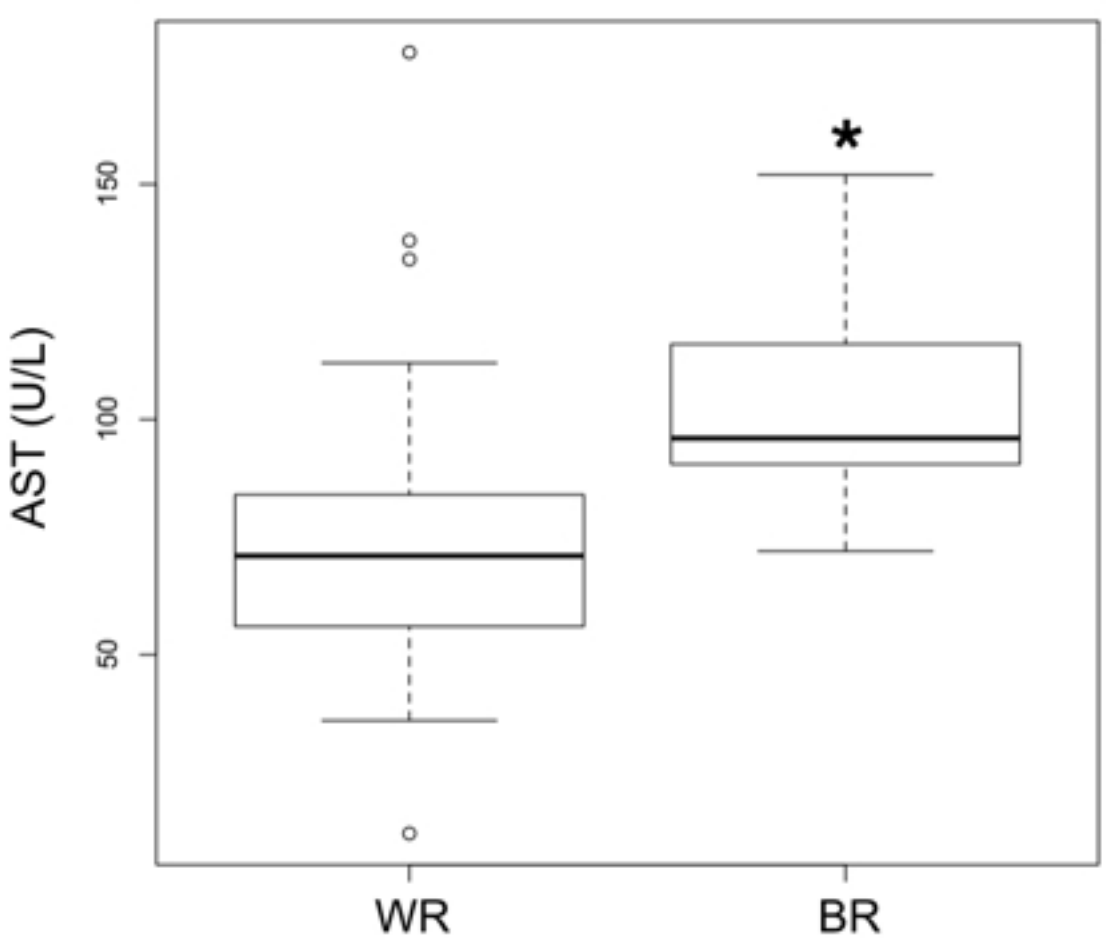
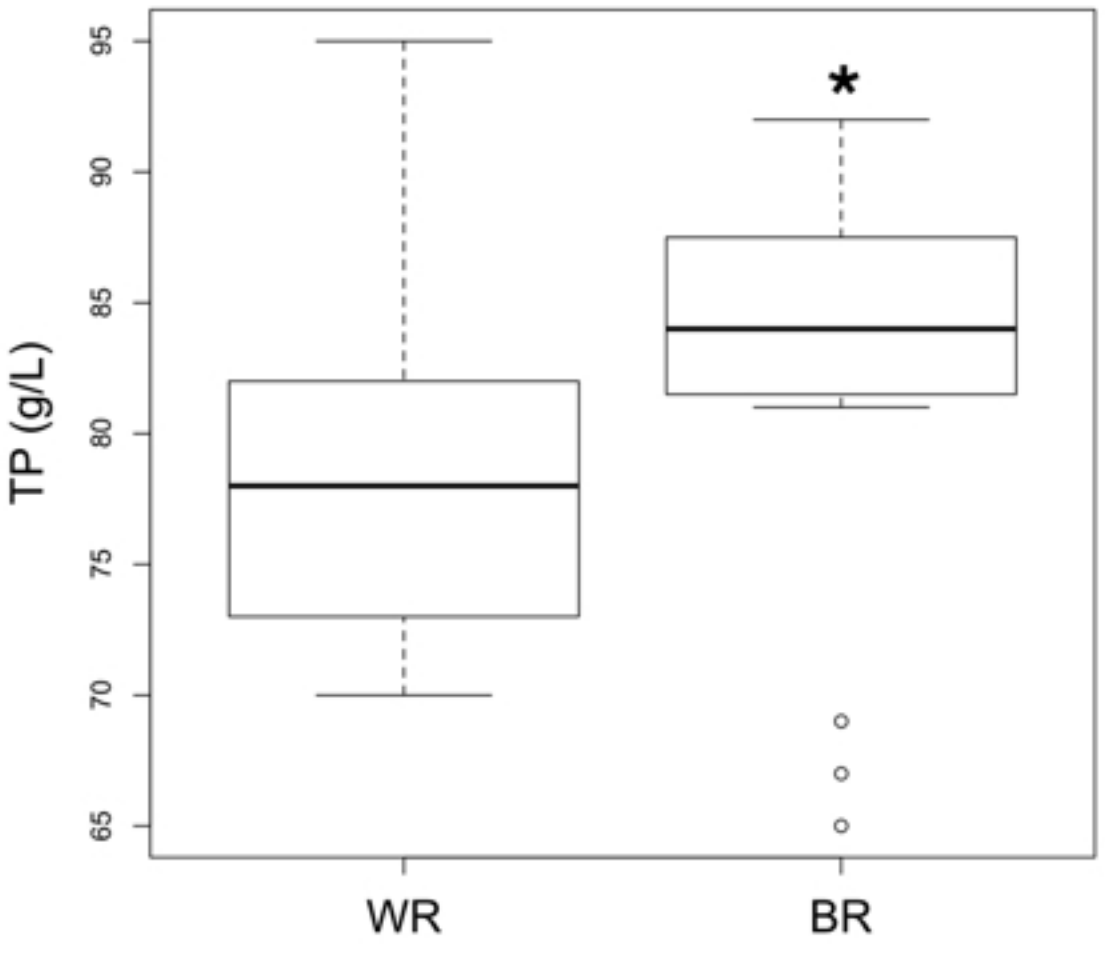
WR
BR


AST (U/L)
50
100
150
WR
BR


TP (g/L)
65
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WR
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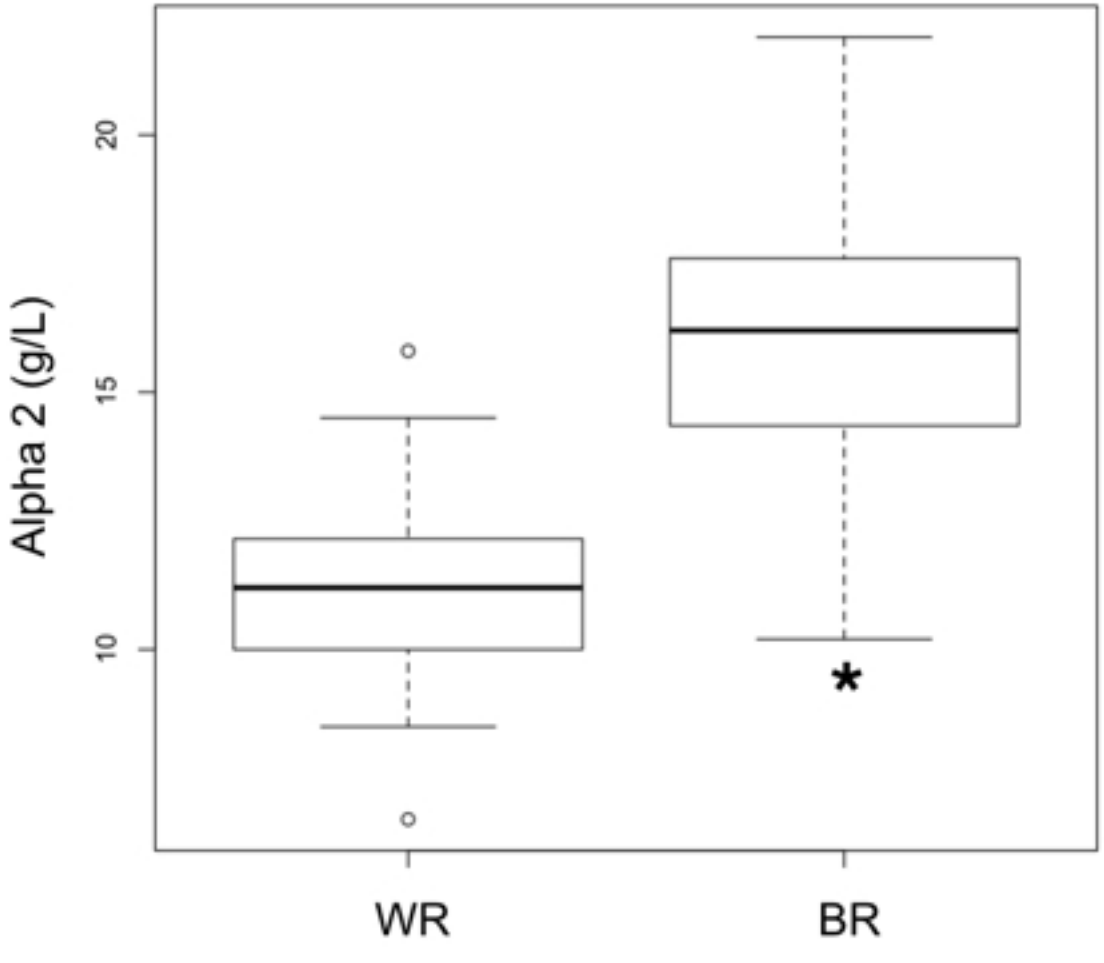
Alpha 2 (g/L)
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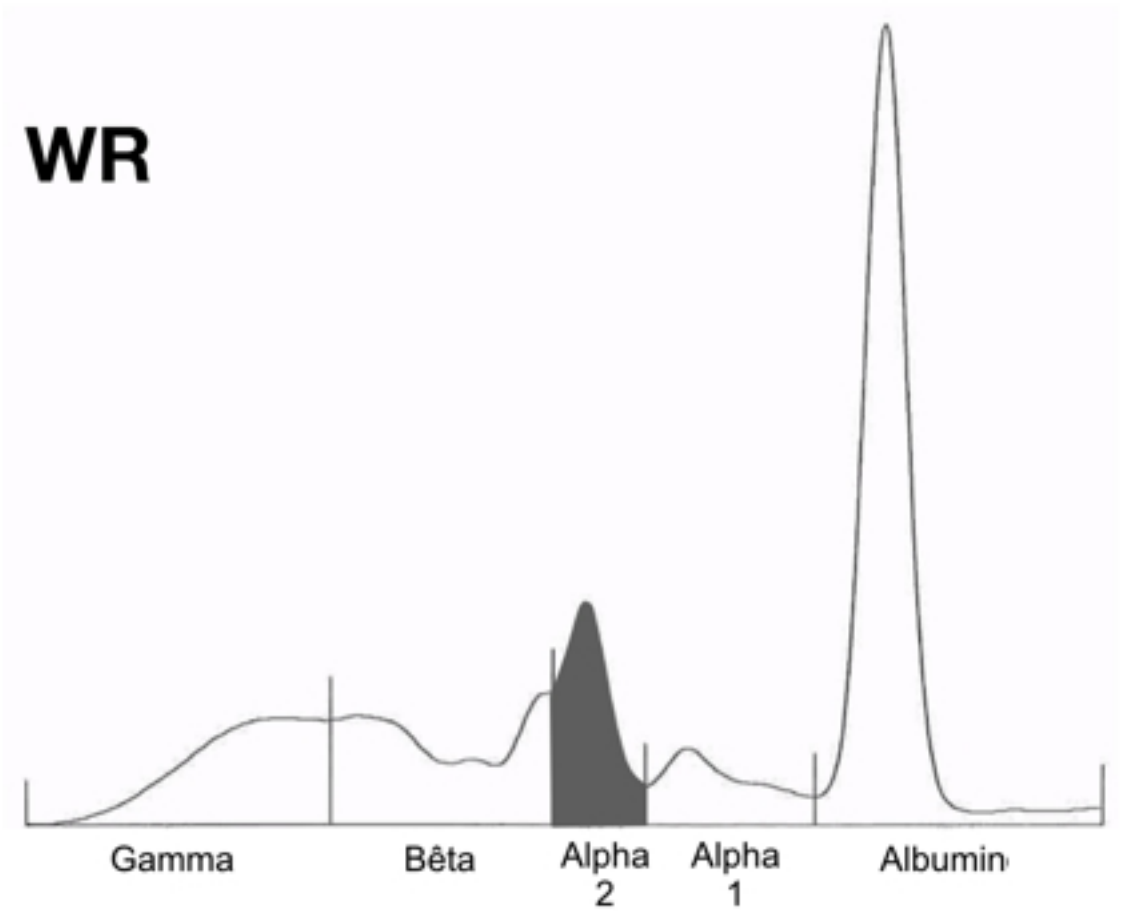
WR
BR

Fig1


WR
Gamma
Bêta
Alpha 2
Alpha 1
Albumin

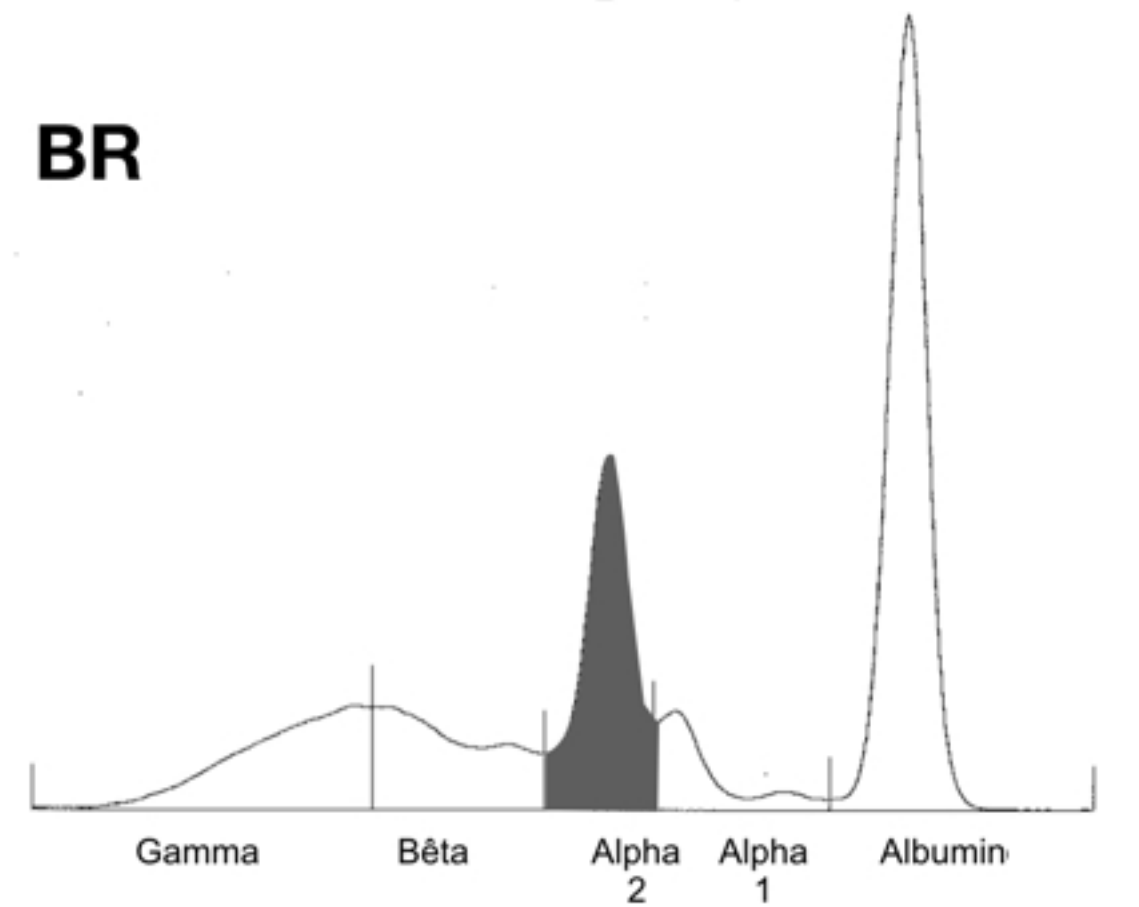

BR
Gamma
Bêta
Alpha 2
Alpha 1
Albumin

Fig2