

## RESEARCH ARTICLE

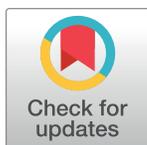
# Inflammatory and oxidative status in European captive black rhinoceroses: A link with Iron Overload Disorder?

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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 develops 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 authors hypothesized that inflammation and oxidative stress may be implicated in the pathogenesis of IOD in captive black rhinoceroses, making this syndrome a potential common denominator to various diseases described in captivity in this species. 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 dROMs ( $P < 0.01$ ) and a trend for higher GPX ( $P = 0.06$ ) levels. The albumin/globulin ratio was lower in black rhinoceroses ( $P < 0.05$ ) due to higher  $\alpha_2$ -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. 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.

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## Introduction

Black rhinoceroses (*Diceros bicornis*, BR) are browsers 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 natural diet and the diets fed in captivity, which may lead to increased availability of iron in the latter [6,11,13–16].

In humans, hemochromatosis is considered as an inflammatory disease [17] with increased oxidative stress [18]. Oxidative stress has severe consequences on health through high tissue and cellular toxicity [17–22], thus participating in cancer formation [19,23] and promoting secondary diseases as well as 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 [18,24]. 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 [18]. This interaction has already been suggested in a BR, since they seem to experience a high susceptibility to oxidative stress compared to other mammals [25–30] 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

Blood samples of 15 BR from 8 European zoological institutions (9 females and 6 males, aged 5–33 yr-old, average age of 19 yr-old) and 29 WR from 14 European zoological institutions (18 females and 6 males, five unknown, aged 4–46 yr-old, average age of 16 yr-old) were included in the study between May 2017 and May 2018. All of these institutions are members of the *European Association of Zoos and Aquaria* (EAZA) and participate in the BR or WR *European Endangered Species program* (EEP). This prospective study was validated and supported by coordinators of both BR and WR EAZA's EEP. All blood samples were collected during routine blood sampling for health monitoring, during an anaesthesia procedure or a medical training session that was planned to occur independently from the present study, and leftover specimens were donated to this study. Therefore, the obtainment of ethical approval was not required.

Diet of BR mainly included alfalfa hay, browses, fruits, vegetables, and herbivore pellets. Diet of WR mainly included hay, grass and grazer pellets. In 5/16 WR for which information

about the diet was available, vitamin E complements was also offered. None of these captive BR and WR were reported to receive iron chelators. 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.

### Sample collection, processing and analysis

Three millilitre blood samples were collected from the auricular or radial vein of each BR and WR, 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. Samples were kept less than four days at +4°C until shipment, and then sent to the veterinary laboratory (LDHVet-LabOniris, Nantes, France), at ambient temperature. 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 [31], the hepatic and muscular functions were investigated through the measurement of the following parameters: aspartate aminotransferase (AST, L-aspartate/ $\alpha$ -oxoglutarate as substrate, on serum), gamma glutamyltransferase (GGT, L- $\gamma$ -glutamyl-3-carboxy-4-nitroanilide/glycylglycine as substrate, on serum), cholesterol (cholesterol esterase/oxidase method, on serum) and creatine kinase (CK, creatine phosphate as substrate, on serum). Inflammation status was evaluated by measuring total serum protein (TP, biuret method) and agarose gel electrophoretic serum albumin and globulin fractions (albumin and globulins including  $\alpha_1$ -,  $\alpha_2$ -,  $\beta$ - and  $\gamma$ -globulin fractions, agarose gel method, Hyrys2, Hydragel; Sebia, Evry, France). Globulin fractions were determined according to recent published data from Hooijberg and colleagues [32].  $\alpha_1$ -globulins refers to  $\alpha_1$ -a and  $\alpha_1$ -b fractions, and  $\beta$ -globulins refers to  $\beta_1$  and  $\beta_2$  fractions. The albumin:globulin ratio (A/G) was calculated. Finally, oxidative stress was assessed through the measurement on heparinized whole blood 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: this information is missing for two BR and three WR for which the serum received was not of sufficient quantity. 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 [33]. 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, GGT, SOD, dROMs and GPx) in order to compare BR and WR. For statistical comparison, results that fall outside the assays' ranges were given a fixed value, maximum measurable value +1 or minimum measurable value -1 for high and low results respectively. Statistical significance was set at  $P < 0.05$ .

## Results

Measured parameters for both European captive BR and WR are listed in Table 1. Sex distribution did not differ between BR and WR ( $P = 0.32$ ), nor did age ( $P = 0.32$ ). Serum iron was higher ( $P < 0.01$ ) in BR (Fig 1). Regarding liver and muscular function, AST activity (Fig 1,  $P < 0.01$ ), GGT ( $P < 0.0001$ ) and CK ( $P < 0.01$ ) were higher in BR, as were total proteins ( $P < 0.05$ ; Fig 1). The A/G ratio was lower ( $P = 0.01$ ) in BR because of higher ( $P < 0.001$ ) levels of  $\alpha_2$ -globulin (Figs 1 and 2). Finally, regarding oxidative stress assessment, dROMs ( $P < 0.01$ ) were significantly higher in BR. A trend ( $P = 0.06$ ) was also observed for GPX with higher values observed in BR.

## Discussion

The objective of this prospective study was to investigate the possible role of inflammation and oxidative stress in the pathogenesis of Iron Overload Disorder in European captive BR. Results showed that European captive BR exhibited higher serum iron concentration and higher inflammatory and oxidative status than captive white rhinoceroses. Taken together, these

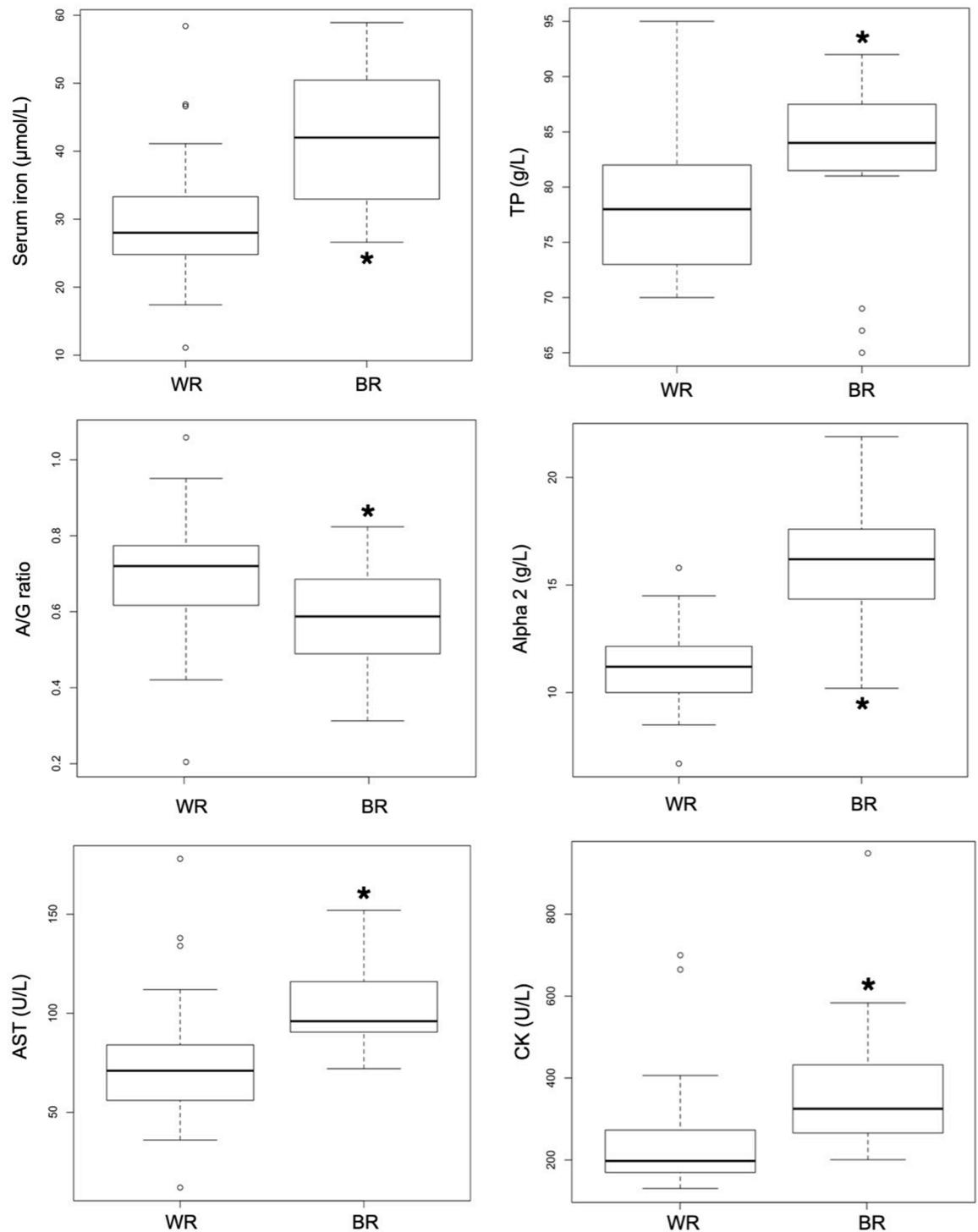
**Table 1. Results for parameters measured in European captive black and white rhinoceroses for the evaluation of iron status, hepatic and muscular function, inflammation status and oxidative stress levels.**

	Black rhinoceros						White rhinoceros					
	Mean	SD	Min	Median	Max	n	Mean	SD	Min	Median	Max	n
Age (years)	19	9.2	5	21	33	15	16	10.0	4	16	46	26
Serum iron ( $\mu\text{mol/L}$ )	42.2	10.7	26.6	42.0 <sup>a</sup>	58.9	15	29.8	9.9	11.1	28.0	58.4	29
AST (U/L)	104	21.4	72	96 <sup>a</sup>	152	15	77	33.4	12	71	178	29
CK (U/L)	379	201.7	199	323 <sup>a</sup>	945	13	246	142.9	129	196	697	26
GGT (U/L)	25	8.1	14	24 <sup>a</sup>	45	15	NC	NC	< 8	10	20	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 <sup>a</sup>	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 <sup>a</sup>	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
$\alpha_1$ -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
$\alpha_2$ -globulin (g/L)	15.8	3.0	10.2	16.2 <sup>a</sup>	21.9	15	11.3	1.9	6.7	11.2	15.8	28
$\beta$ -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
$\gamma$ -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
GPX (U/g Hb)	324	134.2	104	346	560	14	240	113.8	47	239	423	28
dROMs (U CARR)	NC	NC	416	978 <sup>a</sup>	> 1,000	15	NC	NC	380	686	> 1,000	28

AST, aspartate aminotransferase; CK, creatine kinase; GGT, gamma glutamyltransferase; TP, total serum protein; A/G ratio, albumin/globulin ratio; SOD, superoxide dismutase; GPX, glutathione peroxidase; dROMs, reactive oxygen metabolites; NC, not calculated parameter, because of values out of measurable assay range.

<sup>a</sup> Statistically significant ( $P < 0.05$ ) differences between black rhinoceroses and white rhinoceroses.

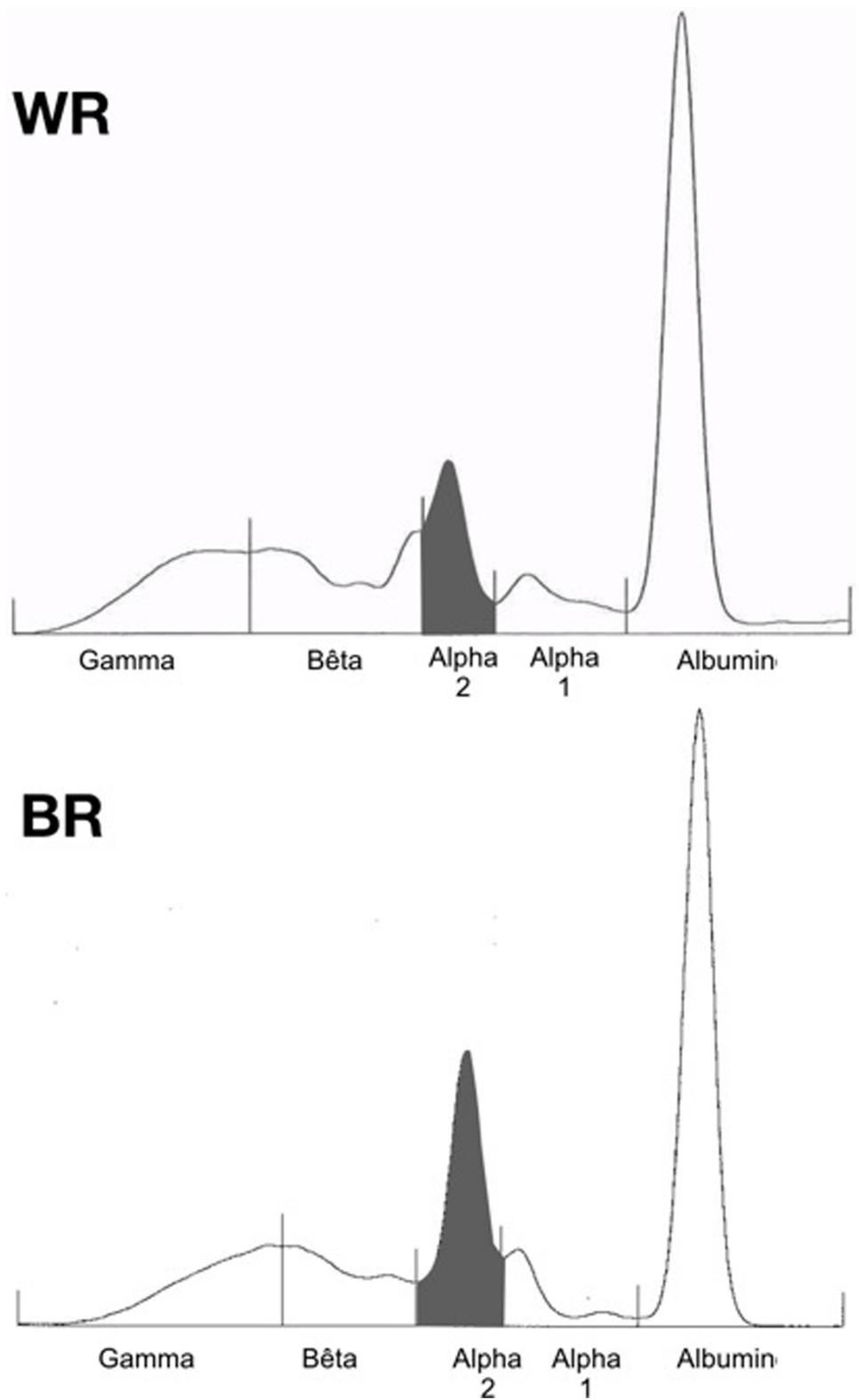
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**Fig 1.** Boxplots showing the quartiles and outliers of serum iron, TP, A/G ratio,  $\alpha_2$ -globulins AST, and CK results in 15 European captive black rhinoceroses (BR) and 29 white rhinoceroses (WR). Values were significantly different between the species in all cases ( $P < 0.05$ ).

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findings corroborate the concept that BR could be predisposed to iron accumulation probably leading to IOD and enhancing inflammatory and oxidative states.



**Fig 2. Serum protein electrophoreses showing an example of the serum protein distribution in a black rhinoceros (BR, total proteins 92 g/L,  $\alpha_2$  globulins 27 g/L) and a white rhinoceros (WR, total proteins 75 g/L,  $\alpha_2$  globulins 9.6 g/L). Note the increased size of the  $\alpha_2$  globulin region in the BR compared to the WR (grey areas).**

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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 [14,34] captive BR. Serum iron levels being higher in captive BR compared to captive WR has already been reported [8,34]. These results confirm a predisposition of captive European BR to develop IOD, as previously described [35]. Even if liver biopsy remains the gold standard for definitive diagnosis of iron overload syndromes [36] and has already been performed in a live captive BR confirming diffuse hemosiderosis [37], 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 [8,38], ferritin is not specific for iron overload syndromes [39] and is reported as a poor biomarker for IOD progression in Sumatran as well as in black rhinoceroses [40,41]. 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 [10,34,35,37] 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 (TS) 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 [42]. We hypothesize that transferrin from rhinoceroses is 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. Pre-analytical factors (shipment, sample preparation and conditioning) may also have skewed this assay. Other methods, such as evaluation of unbound iron binding capacity (UIBC) might be more suitable to calculate Transferrin Saturation in rhinoceroses, as reported in other studies testing rhinoceroses [43,44]. Further studies are needed to establish the ideal method to obtain TS in rhinoceroses.

Liver function was assessed through AST and GGT measurements, as recommended for domestic horses [45], especially when investigating hemochromatosis [46,47]: indeed, the horse is considered as a very good domestic model animal for rhinoceroses [16,48]. 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 [17,31]. Iron deposition in muscles has been reported in BR affected by IOD [31]. 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 [49–51], 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  $\alpha_2$ -globulin fraction were observed in captive BR compared to WR, highly suggestive of an increased inflammatory state [52], as it is described in humans affected by hemochromatosis [17,53]. In this study, selecting captive WR instead of free-ranging BR as the negative control aimed at eliminating the captivity bias since captivity may be pro-inflammatory by itself [38,54]; however, differences in the

reaction to captivity between the two species may occur. Serum protein electrophoretic results of the captive WR included in this study showed some variations in comparison to healthy free-ranging WR [32], 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 [55], but no study to date investigates the consequences of overweight on inflammation 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. The main proteins migrating in the  $\alpha_2$  region on serum protein electrophoresis include haptoglobin,  $\alpha_2$ -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  $\alpha_2$ -globulin increase in the present study.  $\alpha_2$ -macroglobulin inhibits numerous endogenous proteases and acts as a transport protein for cytokines and growth factors [56]. Increased  $\alpha_2$ -macroglobulin is favoured by inflammatory states, such as diabetes mellitus in humans [56]. Ceruloplasmin levels increase with inflammation in humans [57]. Among many roles, this ferroxidase helps to reduce circulating free ferrous iron [18]. 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. Hence, its measurement could be of interest in future studies on IOD in BR. Finally, SAA, which increases during inflammation, was reported to be significantly higher in captive BR compared to wild BR [54]. As a result, an increase in  $\alpha_2$ -macroglobulin, ceruloplasmin and SAA, which all suggest inflammation, could explain the increase in  $\alpha_2$ -globulin observed in the captive BR in this study. This finding suggests that IOD could be linked to a chronic inflammatory disease like hemochromatosis in humans [17]. 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 [58]. 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, e.g. with regular phlebotomies as previously described [59].

dROMs are hydroperoxydes, meaning reactive oxygen metabolites that allow to directly evaluate oxidative stress levels [60,61]. SOD and GPX are antioxidant enzymes whose activity measurement indirectly allows to quantifying the response to oxidative stress [62,63]. GPX enzymes are key regulators of cellular lipid peroxides, which are an important class of reactive oxygen species [64]. In the present study, significantly higher levels of dROMs, and a trend for higher levels of GPX, 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 [26,29,30], captivity itself [38,54] and/or a self-sustaining process in which oxidative stress disrupts antioxidant defences [62]. High levels of oxidative stress can predispose to diseases and rapid ageing [18] 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 [18]. 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.

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. The hypothesized link between increased inflammation and oxidative stress levels and the development of IOD in captive BR needs further investigation. 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  $-20^{\circ}\text{C}$  [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.

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## Author Contributions

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**Supervision:** Rudy Wedlarski, Laetitia Jaillardon.

**Validation:** Nicolas Soetart, Delphine Boucher, Laetitia Jaillardon.

**Visualization:** Nicolas Soetart.

**Writing – original draft:** Hanae Pouillevet.

**Writing – review & editing:** Hanae Pouillevet, Nicolas Soetart, Rudy Wedlarski, Laetitia Jaillardon.

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