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Research article

Rhinoceros serum labile plasma iron and associated redox potential: interspecific variation, sex bias and iron overload disorder disconnect

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A consequence of the poaching crisis is that managed rhinoceros populations are increasingly important for species conservation. However, black rhinoceroses (BR; *Diceros bicornis*) and Sumatran rhinoceroses (SR; *Dicerorhinus Sumatrensis*) in human care often store excessive iron in organ tissues, a condition termed iron overload disorder (IOD). IOD research is impeded by the challenge of accurately monitoring body iron load in living rhinoceroses. The goals of this study were to (i) determine if labile plasma iron (LPI) is an accurate IOD biomarker and (ii) identify factors associated with iron-independent serum oxidative reduction potential (ORP). Serum (106 samples) from SRs ($n = 8$), BRs ($n = 28$), white rhinoceros ($n = 24$) and greater one-horned rhinoceros (GOH; $n = 16$) was analysed for LPI. Samples from all four species tested positive for LPI, and a higher proportion of GOH rhinoceros samples were LPI positive compared with those of the other three species ($P < 0.05$). In SRs, the only LPI-positive samples were those from individuals clinically ill with IOD, but samples from outwardly healthy individuals of the other three species were LPI positive. Serum ORP was lower in SRs compared with that in the other three species ($P < 0.001$), and iron chelation only reduced ORP in the GOH species ($P < 0.01$; ~5%). Serum ORP sex bias was revealed in three species with males exhibiting higher ORP than females ($P < 0.001$), the exception being the SR in which ORP was low for both sexes. ORP was not associated with age or serum iron concentrations ($P \geq 0.05$), but was positively correlated with ferritin ($P < 0.01$). The disconnect between LPI and IOD was unanticipated, and LPI cannot be recommended as a biomarker of advanced rhino IOD. However, data provide valuable insight into the complex puzzle of rhinoceros IOD.

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Introduction

Many wildlife species in human care, ranging from toucans to dolphins, store excessive iron as hemosiderosis in organ tissues (Claus and Paglia, 2012). Within the Rhinocerotidae family, this condition is prevalent in both black rhinoceros (BR) and Sumatran rhinoceros (SR) tissues examined post-mortem (Kock *et al.*, 1992; Olias *et al.*, 2012; Paglia and Tsu, 2012). However, the aetiology of iron storage and the extent of its impact on rhinoceros health are obscure, likely complex and not analogous to that of hemochromatosis, the most common iron storage disease in humans caused

by genetic mutations. Therefore, the rhinoceros condition has been deemed iron overload disorder (IOD) until it is better understood and its repercussions known (Citino *et al.*, 2012). Although often referred to as a browsing rhinoceros disorder since BRs and SRs are browsers (Hall-Martin *et al.*, 1982; van Strien, 1986), IOD and associated hemosiderosis have now also been reported in greater one-horned (GOH) rhinoceros (Ball and Mueller, 2011; Olias *et al.*, 2012), which are generally grazers (Hazarika and Saikia, 2012).

Despite the hemosiderosis and high concentrations of iron measured in BR and SR livers (Dierenfeld *et al.*, 2005; Roth *et al.*, 2017), IOD is rarely identified as the primary cause of death in rhinoceroses (Dennis *et al.*, 2007; Roth *et al.*, 2019a). Regardless, it seems logical that such high iron loads in vital organs could be detrimental to the health of these species. Excessive iron deposits could damage cells directly via mechanical cell structural disruption, but more likely do so indirectly via one or more physiological pathways. For example, because iron is a potent catalyst of the Fenton reaction, its production of reactive oxygen species (ROS) could over time cause cumulative, wide-spread cellular trauma leading to organ tissue damage that results in cancer, fibrosis or other life-threatening pathologies (Cabantchik *et al.*, 2005). Alternatively, high body iron load could exacerbate susceptibility to diseases caused by iron-dependent, pathogenic microbes typically kept in check via stiff competition for scant bioavailable iron (Cassat and Skaar, 2013; Cronin *et al.*, 2019; Roth *et al.*, 2019b). Furthermore, iron dysregulation can interfere with systemic and local immune function via numerous intricate pathways (Cronin *et al.*, 2019; Mu *et al.*, 2021). Although studies of immune cell function (Roth and Vance, 2007; Vance *et al.*, 2004), gut microbiome (Roth *et al.*, 2019b; Williams *et al.*, 2019) and serum ROS (Paglia *et al.*, 1996; Pouillevet *et al.*, 2020) have revealed differences among rhinoceros species, evidence directly linking body iron load to impaired health is lacking.

The study of IOD in living rhinoceroses is seriously impeded by the inability to accurately monitor body iron load. Iron concentrations in liver biopsies provide the gold standard for measuring systemic iron status, but such biopsies are risky and challenging to obtain from rhinoceroses. Serum ferritin has long been used as an indicator of iron status in a variety of species (Lipschitz *et al.*, 1974; Smith *et al.*, 1984a,b; Weeks *et al.*, 1989; Andrews *et al.*, 1994), but it is an acute phase protein not specific to iron (Lee and Means, 1995; Wang *et al.*, 2010). Furthermore, ferritin can be species-specific with variable or no cross-reactivity to antibodies made against ferritin of another species (Richter, 1967; Roth *et al.*, 2017), and in studies that employed rhinoceros ferritin-specific monoclonal antibodies, serum ferritin proved unreliable for monitoring iron load in rhinoceroses (Roth *et al.*, 2017; Wojtusik and Roth, 2018). Even in humans, ferritin's efficacy as an iron bioindicator has known shortcomings and is not universally effective, although it can be useful under certain conditions (Wood, 2014). Percent transferrin saturation is often considered another useful marker of body iron status, but its usefulness also varies depending on the aetiology of iron loading (Wood, 2014). Furthermore, it has proven technically difficult to measure accurately in rhinoceroses (Pouillevet *et al.*, 2020; personal experience). Potential alternative serum biomarkers include non-transferrin bound iron (NTBI), which has received considerable attention in the human health field (Zhu *et al.*, 2016). NTBI can include a small fraction of labile plasma iron (LPI), which is typically present only in states of severe chronic or acute iron overload and is proving valuable as a biomarker of iron status in hemochromatosis patients undergoing chelation therapy (Esposito *et al.*, 2003; Pootrakul *et al.*, 2004; Zhu *et al.*, 2016). LPI is especially damaging because it is highly redox active, capable of permeating into organs and catalysing the formation of ROS in tissues (Cabantchik *et al.*, 2005).

The goals of this study were to (i) determine if non-transferrin bound LPI could serve as an accurate biomarker of IOD since LPI should only be present in individuals with high body iron loads and (ii) test factors (species, iron, IOD, age, sex and ferritin) potentially associated with the oxidative reduction potential (ORP) measured in the LPI assay.

Materials and methods

Ethics statement

All activities in this study were approved by the Cincinnati Zoo and Botanical Garden's Institutional Animal Care and Use Committee under protocol #19-154 entitled 'Non-invasive and harmless opportunistic collection of biological samples from animals'. Serum samples for this research were either stored frozen (−20°C or −80°C) in the rhinoceros serum bank at the Center for Conservation and Research of Endangered Wildlife (CREW) or were sent frozen to CREW from serum banks at other facilities that agreed to provide samples for the study.

Study 1 animals and samples

In Study 1, serum samples ($n = 20$) from 8 BRs (6 males and 2 females; age range, 6–29 y; mean age, 19.5 y) and 8 SRs (22 samples from 2 males and 6 females; age range, 2–30 y; mean age, 17.7 y) were analysed for LPI. Samples were chosen from asymptomatic, outwardly 'healthy' rhinoceroses (33 samples from all 16 individuals), rhinoceroses diagnosed with IOD post-mortem based on liver iron content and histopathology that were symptomatic at the time of sampling ('IOD symptoms'; 4 samples from 3 individuals) or asymptomatic 1–2 y prior to death ('pre IOD symptoms'; 3 samples from 3 individuals) and rhinoceroses exhibiting symptoms of serious illness that were diagnosed with an unrelated disease and cause of death post-mortem 2–4 weeks after sampling ('other illness'; 2 samples from 2 individuals). Multiple samples within individuals represent different times and/or different

'health status' in the rhinoceroses' life and enable individuals to serve as their own controls when healthy versus sick. A minimum of one and maximum of five samples/individual were analysed.

Study 2 animals and samples

No additional samples from SRs were analysed for Study 2, but the number of BR samples increased [44 total samples from 14 males (23 samples) and 14 females (21 samples) ranging in age from 1–38 y; mean, 16.1 y] and both white rhinoceros (WR) samples (24 total from 12 males and 12 females ranging in age from 3–40 y; mean, 18.0 y) and GOH rhinoceros samples (16 total from 8 males and 8 females ranging in age from 3–34 y; mean, 14.1 y) were included. Because a sex effect on ORP became apparent early in the study, the number of males and females represented within each of the three species was balanced. BR samples were assigned to ferritin concentration categories of low (<1000 ng/ml), moderate (>4000 but <10 000 ng/ml) and high (>10 000 ng/ml) based on data from a previous study (Wojtusik and Roth, 2018), and approximately equal numbers of samples were included within each category (15, 12 and 14, respectively). Because many samples had previously been thawed, a freeze–thaw test was conducted using two aliquots each of two serum samples that were thawed for the first or sixth time on the day of the assay.

LPI assay

LPI was measured using the assay aFerrix FeROS™ LPI kit (Aferix Ltd, Kiryat Atidim, Tel-Aviv, Israel) because of its reported specificity for measuring only LPI iron instead of all NTBI present in serum (Zhu *et al*., 2016). The assay measures redox generated via the iron-induced Fenton reaction over time. ORP is quantified using the oxidation-sensitive probe, dihydrorhodamine 123, and the change in fluorescent units/minute (ΔFU/min) is calculated from fluorometer readings taken every 2 min for 20 consecutive minutes. Sample testing was conducted in a 96-well plate, and each sample was added to four wells; duplicate wells that contained an iron chelator, and duplicate wells without the chelator. Fluorometer readings were taken using a Synergy2 microplate reader (BioTeck). The difference in ORP between the two sets of wells was the amount of ORP attributed to LPI. Positive and negative LPI controls were supplied with the kit for quality control and for use when calculating the LPI unit values according to the LPI equation template provided with the assay. Only values >0.20 were considered LPI-positive samples in accordance with the assay instruction manual (TSL902 V.11). In one final assay, a copper chelator (tetrathiomolybdate; TTM) (Juarez *et al*., 2006; Nguyen *et al*., 2014) was substituted for the iron chelator to determine if copper was responsible for inducing the Fenton reaction and resulting redox in some samples. A high concentration of 1 mM TTM was tested on a subset of 31 BRs ($n = 23$) and GOH rhinoceros ($n = 8$) samples.

Serum iron analysis

To determine if there was a relationship between serum iron concentration and LPI or ORP, a subset of rhino serum samples ($n = 70$) was analysed for total iron content using a colourimetric assay (BioVision, Milpitas, CA, USA). Samples from BR (26 from 15 males and 11 females), WR (17 from 10 males and 7 females), GOH rhinoceros (13 from 8 males and 5 females) and SR (14 from 3 males and 8 females) were chosen based on residual sample volume available for testing while ensuring a mix of both LPI-positive and -negative samples and male and female samples within each species. Across all species, a total of 21 LPI positive and 49 LPI negative samples were evaluated for iron concentration.

Statistical analyses

For all statistical tests, a $P < 0.05$ was considered significant. A Fisher exact test was used to compare proportions of positive LPI samples among species and among high, moderate and low ferritin groups within BR samples. Furthermore, Spearman's rho correlation coefficients were determined for the relationships between ferritin concentration and ORP in BRs and between age and ORP across all species. A one-tailed, paired *t*-test with Wilcoxon correction was used within each species to determine if sample ORP decreased in the presence of an iron chelator. Finally, the entire ORP data set was analysed using the statistical programming language R ver. 1.4.1717 (R Development Core Team, 2019). Data failed the Shapiro–Wilk normality test ($P < 0.001$), and variance was not equal among species or sex, so generalized linear mixed effects models were tested, and the negative binomial distribution model proved a good fit. Species, sex and species∗sex interaction were all main effects in the model with *post hoc* testing performed using estimated marginal means and Tukey's pairwise comparisons.

Total serum iron was compared among species and to LPI status using a one-way analysis of variance (ANOVA) with species or LPI status as fixed factors and serum iron the dependent variable. Data passed Levene's test for equality of variance, and *post hoc* testing was performed using Tukey's test. The relationship between ORP and serum iron concentrations was assessed using linear regression analysis. Because SR ORP values were so much lower than those of the other rhino species, the analyses were performed twice, once with all rhino species included and again with SR samples excluded.

Results

Study 1

LPI test results

LPI-positive samples were observed in both SR and BR species (Table 1). In SRs, the only LPI-positive samples were those collected from rhinoceroses ill with IOD. In contrast, a significantly higher proportion (7/17; $P < 0.05$) of samples collected

Table 1: Study 1 LPI testing results for serum from BRs and SRs of different health status

Rhinoceros species	Rhinoceros health[a] (n = number of rhinos)	LPI positive	LPI negative
BR	Healthy ($n = 8$)	7 (n = 5)	10 ($n = 6$)
	Pre-IOD symptoms ($n = 1$)	0	1
	IOD symptoms ($n = 1$)	1	0
	Other illness ($n = 1$)	0	1
SR	Healthy ($n = 8$)	0	16 ($n = 8$)
	Pre-IOD symptoms ($n = 2$)	0	2 ($n = 2$)
	IOD symptoms ($n = 2$)	3 ($n = 2$)	0
	Other illness ($n = 1$)	0	1

[a]Pre-IOD symptoms, 1–2 y prior to death associated with IOD; IOD symptoms, symptoms of illness followed by IOD diagnosis post-mortem; other illness, symptoms of illness followed by diagnosis of non-IOD related cause of death post-mortem. In all columns, numbers represent serum sample count; n, number of individuals contributing to the sample count.

Healthy, no symptoms of illness.

from healthy BRs tested positive for LPI, with four of eight individuals sampled on multiple dates producing a mix of negative and positive LPI results. Samples collected from BRs and SRs 1–2 y prior to IOD-related mortality were not positive for LPI. Similarly, samples collected 2–4 weeks prior to death from non-IOD-related causes were LPI negative. Of the two samples used for the freeze–thaw testing, one was LPI positive (both aliquots) and one was LPI negative (both aliquots), and ORP increased slightly in one and decreased slightly in the other after six thaws. In either case, the difference in ORP between aliquots thawed 1× or 6× was no more than 10%.

Study 2

LPI test results

Although some samples from all four rhinoceros species tested positive for LPI, a higher proportion of GOH rhinoceros samples were LPI positive compared with that for the other three species ($P < 0.05$; Fig. 1). In total, 13 male and 15 female samples were LPI positive indicating no significant sex bias. Data were also analysed after removing the six samples from symptomatic animals. The only change in significance was a difference between BR and SR with the former having significantly more LPI-positive samples.

Oxidative reduction potential (oxidation rate)

High ORP was detected by DHR oxidation rate in serum from three of the four rhinoceros species (BR, WR and GOH), and the iron chelator had minimal to no effect in blocking ORP in these samples. Only serum from GOH rhinoceros exhibited significantly less ORP in the presence of the iron chelator compared with the control samples ($P < 0.01$), but that difference accounted for only 5% of the total ORP measured. In contrast, BR and WR serum ORP trended slightly higher in the presence of the iron chelator. Although ORP was very similar in most samples with or without the iron chelator, for consistency, all subsequent statistical analyses were conducted using ORP values in samples with iron chelator which should represent ORP unrelated to serum iron.

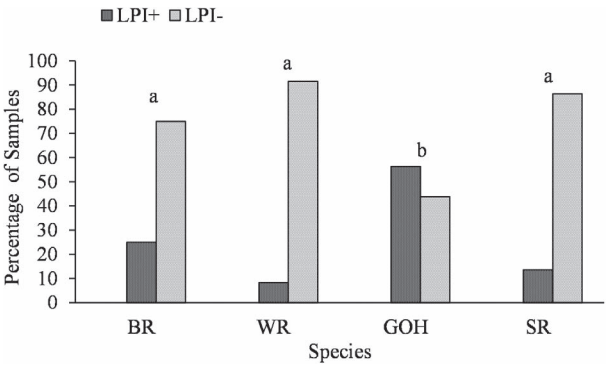

Figure 1: Proportion of samples testing positive and negative for LPI within each rhinoceros species (BR, 44 samples from 28 individuals; WR, 24 samples from 24 individuals; GOH, 16 samples from 16 individuals; SR, 22 samples from 8 individuals). Different letters denote differences in the proportion of LPI-positive samples among species (Fisher exact test, $P < 0.05$).

When the copper chelator TTM was tested on a subset of samples, it turned the medium a light copper colour, which quenched the fluorescence detected in chelator-treated wells. Therefore, comparisons between control wells without chelator and the chelator-treated wells were confounded. However, based on the changing values over time in the chelator-treated wells, some DHR oxidation appeared to be occurring despite the presence of high copper chelator concentration.

Both species and sex were significant factors impacting ORP in rhinoceros serum ($P < 0.001$; Figs 2 and 3). Mean ORP in GOH and BR serum was about twice as high as it was in WR serum, but WR mean ORP was >20X higher than that

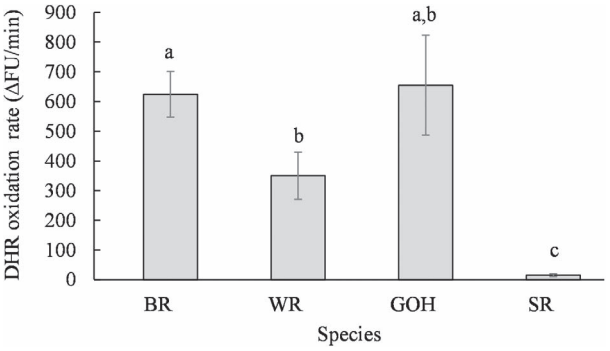

Figure 2: DHR oxidation rates (means ± SEM) for each rhinoceros species (BR, 44 samples from 28 individuals; WR, 24 samples from 24 individuals; GOH, 16 samples from 16 individuals; SR, 22 samples from 8 individuals). Different letters denote differences between species (generalized linear mixed effects model with *post hoc* testing, $P < 0.05$).

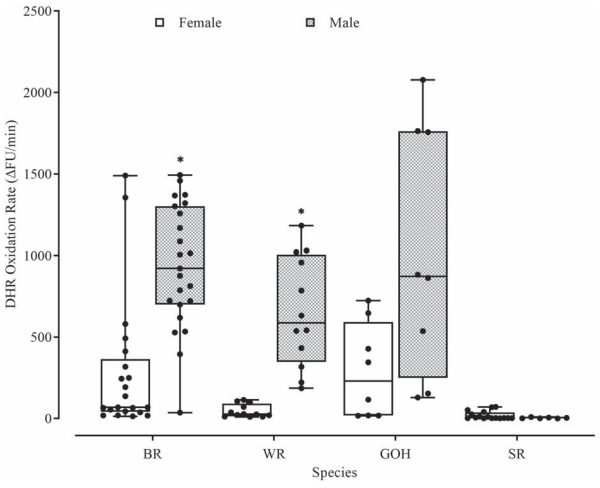

Figure 3: DHR oxidation rates (means ± SEM) by sex within each rhinoceros species. Asterisks denote differences between sexes within species (generalized linear mixed effects model with *post hoc* testing, $P < 0.05$).

for SR serum (Fig. 2). Statistically, SR ORP was lower than that in all three other species ($P < 0.001$), BR ORP was greater than that for WRs ($P < 0.05$), but the difference between GOH rhinoceros and WR mean ORP did not quite achieve significance ($P = 0.068$) due to the smaller GOH sample size. The analysis was repeated after excluding samples from symptomatic animals ($n = 6$) and the only change in results was a lower *P*-value ($p < 0.01$) in the *post hoc* comparison of BRs and WRs. Interspecific ORP differences could not be attributed to variation in sample storage time because sample storage time was very similar across species ($P = 0.938$; mean values: BR = 12.0 y, WR = 11.8 y, GOH rhinoceros = 11.4 y and SR = 12.5 y).

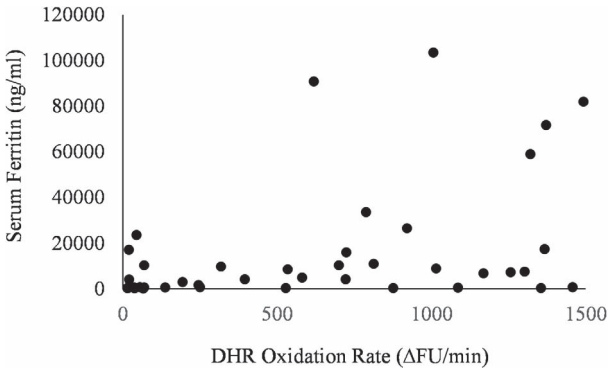

Figure 4: Scatter plot depicting the positive correlation between serum ferritin concentrations and DHR oxidation rate in BRs (41 samples from 25 individuals; Spearman's rho; $r = 0.47$; $P < 0.01$).

Overall, female samples had much lower ORP compared with male samples ($P < 0.001$; Fig. 3). However, the sex difference in mean ORP was only statistically significant in BRs and WRs in the *post hoc* pairwise comparisons. There was a trend towards significance with the GOH samples ($P = 0.10$), but the smaller sample size reduced testing power and prevented achieving significance. In contrast, ORP was skewed higher in females than in males in the SR species, resulting in a significant species∗sex interaction in the generalized linear mixed effects model.

Serum ferritin and rhinoceros age impacts on ORP

The proportions of BR samples testing positive for LPI in the low, moderate and high ferritin concentration categories did not differ ($P \geq 0.05$) with positive samples identified in each category (1/15, 4/12 and 5/14, respectively). However, there was a positive correlation between serum ferritin concentrations and ORP ($r = 0.47$; $P < 0.01$; Fig. 4), although the relationship was not particularly strong. In contrast, BR age was not a significant factor associated with ORP ($p \geq 0.05$), nor was there a correlation between ORP and age when all data from all rhinoceroses were included in the analysis ($r = 0.05$; $P = 0.58$).

Relationship of serum iron with ORP and LPI status

Serum iron concentration differed among species $P < 0.001$ with concentrations similar ($P > 0.05$) in BRs and SRs [means ± SD; 2.41 ± 0.83 ug/ml (range, 0.23–4.25 ug/ml) and 2.76 ± 1.03 ug/ml (range, 1.59–5.61 ug/ml), respectively] and higher ($P < 0.05$) than those found in WRs and GOH rhinoceros [1.52 ± 0.90 ug/ml (range 0.15–3.20 ug/ml) and 1.64 ± 0.47 ug/ml (range 1.03–2.55 ug/ml), respectively]. LPI status was not a significant factor associated with serum iron concentrations, but there was a trend ($P = 0.07$) towards higher serum iron in samples that were LPI positive versus negative (2.44 ± 1.07 and 1.99 ± 0.89 ug/ml, respectively). Finally, there was no significant relationship between ORP

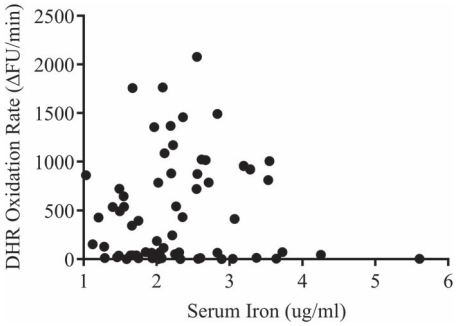

Figure 5: Scatter plot depicting the serum iron concentrations and DHR oxidation rate across all rhinoceros species (70 samples from 26 BRs, 17 WRs, 13 GOHs and 8 SRs; $P = 0.99$).

and serum iron concentration ($P = 0.99$; Fig. 5). Statistical significance did not change in any of the tests after excluding SR samples from the analyses.

Discussion

Quantification of LPI in human serum and its association with iron overload conditions was first reported almost 20 y ago (Esposito *et al.*, 2003), but to our knowledge, this study is the first to test LPI as an IOD indicator in wildlife species. Because IOD is very difficult to diagnose and monitor in rhinoceroses, we relied on serum samples that had been banked years prior to illness and during clinical symptoms from rhinoceroses that had died from IOD or other causes, confirmed post-mortem. Therefore, sample sizes were opportunistic and small in Study 1, but sufficient for determining if LPI shows promise as a rhinoceros IOD biomarker. The only SR serum samples that tested positive for LPI were those obtained from symptomatic individuals sick with IOD, suggesting LPI has potential as a biomarker for late-stage IOD in this species; however, many BR samples were positive for LPI despite the donors' healthy outward appearances. Furthermore, all 11 LPI-positive BR samples were obtained from rhinoceroses that lived at least 5 y and up to 15 y beyond the sampling date, with one exception. A BR with IOD (confirmed post-mortem) died 14 months after the LPI-positive sample was obtained. Together, these data suggest that serum from rhinoceroses sick with IOD is likely to test positive for LPI. However, a high rate of positive tests among BRs that continued living healthy lives for up to 15 y after sampling, suggests LPI testing is not useful for identifying individuals destined to become clinically ill with IOD. Furthermore, for those rhinoceroses that did die of IOD, serum was not positive for LPI 2–3 y prior to death. Only samples collected when these rhinoceroses were showing symptoms of illness tested positive, a point too late in disease progression for corrective treatments to be effective. Because rhinoceroses sick with other deadly illnesses did not test positive, LPI did not appear to be a non-specific response to any severe illness. Although the number of positive samples among outwardly healthy BRs provided some evidence that LPI testing may not be a reliable diagnostic biomarker of progressive IOD, one could argue that virtually all managed BRs store excessive iron and could sporadically test LPI positive. Therefore, the results across all four rhinoceros species included in Study 2 were key in demonstrating LPI's disconnect with IOD in this taxa.

In Study 2, the proportion of LPI-positive GOH samples (56%) was unexpectedly high. The positivity rate for hemochromatosis patients is only 10–15%, so the positivity rate for GOH was closer to that reported for thalassemic patients (~50%) (Esposito *et al.*, 2003). Although GOH and WRs typically have been classified as IOD-resistant species, there are now reports of hemosiderosis and IOD-associated mortality in GOH rhinoceros suggesting that they are susceptible, at least under certain conditions (Ball and Mueller, 2011; Olias *et al.*, 2012). However, despite evidence of hemosiderosis in some GOH liver tissues (Ball and Mueller, 2011; Olias *et al.*, 2012), mineral analyses and histopathology findings both indicate that on average GOH livers contain less iron than those of BRs and SRs (Dierenfeld *et al.*, 2005; Olias *et al.*, 2012). Therefore, a higher proportion of LPI-positive samples from GOH rhinoceros compared with those from BRs and SRs was not anticipated. Furthermore, two WR samples tested positive for LPI despite no reports of excessive hemosiderosis or diagnosed IOD cases in this species to date. These interspecific LPI differences do not align with expectations based on what is known about liver iron loads and rhinoceros susceptibility to IOD and provide further evidence that LPI is not an accurate IOD biomarker in rhinoceros species.

Species differences in serum ORP reflected by the assay DHR oxidation rates were similarly unorthodox. Three of the four species exhibited ORP 20–40 times higher than those reported in healthy human serum (10–15 ΔFU/min) analysed with the same methodology (Esposito *et al.*, 2003). However, specific individuals within each species did exhibit ORP of <20 ΔFU/min indicating the high ORP was not due to some interaction between that species' serum and assay components. Although Esposito *et al.* (2003) did report significantly higher DHR oxidation rates (approaching 100 ΔFU/min) in serum of humans with hemochromatosis, only serum from thalassemic patients was on par with oxidation rates noted in three of the four rhinoceros species (300–600 ΔFU/min) and the oxidation rates in humans dropped substantially (>70%) in the presence of the iron chelator. In contrast, the iron chelator had little to no effect on ORP in rhinoceros serum. In agreement with the LPI findings, only in the GOH species was ORP reduced significantly by the chelator, but that reduction represented only 5% of total ORP. Oddly, SR serum ORP fell within the range recorded for healthy humans, including ORP measured in samples originating from rhinoceroses sick with IOD.

The LPI assay in this study measures the ORP created via the iron catalysed Fenton reaction, and because transferrin-bound iron is redox inactive (Crichton *et al.*, 2002), the

assay is very specific for labile iron. Thus, it was surprising that the iron chelator had little to no effect on reducing ORP in rhinoceros serum. However, copper is also capable of stimulating the Fenton reaction (Kanti Das *et al*., 2014). Therefore, a copper chelator was tested on a subset of BR and GOH rhinoceros samples to determine if labile copper in the serum could be responsible for the ORP. Unfortunately, the copper chelator discoloured the media quenching fluorescence detection and confounding quantitative comparisons to control wells, but it was still apparent that DHR oxidation was occurring in wells containing a very high copper chelator concentration. Since chelation of the two primary Fenton reaction catalysts failed to eliminate ORP in rhinoceros serum, it seems most of the ORP measured is unrelated to the Fenton reaction of the assay. DHR reacts to a wide array of oxidants including hydroxy radicals, hypochlorous acid, peroxynitrites and hydrogen peroxide/peroxidase combinations (Crow, 1997; Gomes *et al*., 2006). It was beyond the scope of this study to identify the source molecules causing high DHR oxidation rates in rhinoceros serum, but it is clearly an intriguing path for future pursuits.

Higher oxidative stress markers in BRs were recently reported in a study that compared BR and WR serum (Pouillevet *et al*., 2020); however, the ratio of males to females within the two species was 6:9 and 6:18, respectively. A strong sex bias was noted early in Study 1 of this project with male rhinoceros samples exhibiting significantly more ORP on average than female samples, so imbalances were largely corrected during Study 2 to avoid influence from the significant confounding factor of sex in the final interspecies analysis. Sex influence on ORP is not surprising since a sex effect on oxidative stress has previously been documented in humans and laboratory animals with oestrogen identified as the mediator of ROS reduction in women and rats (Kander *et al*., 2017; Razmara *et al*., 2007). In rhinoceroses, the sex difference was the most significant factor in the analysis. However, there was also a significant interaction in our analysis because SRs did not follow the sex bias pattern of the other three rhinoceros species. Instead, female SR ORP trended higher than that for males, although the difference was not statistically significant since the sample size was too small. It is possible this trend would disappear with a larger sample size, but no additional individuals were available for sampling. Regardless, serum of both sexes exhibited very low ORP compared with that of the other three rhinoceros species.

Although BR serum exhibited high ORP, SR serum had very low ORP, and prevalence of hemosiderosis is high in both species. Even samples from individuals clinically sick with IOD were not particularly high in serum ORP. Furthermore, this study included GOH rhinoceros samples that contained as much ORP on average as those from BRs. There are case studies demonstrating moderate to significant hemosiderosis in a few GOH rhinoceros (Ball and Mueller, 2011; Olias *et al*., 2012), so it can no longer be considered a strictly IOD-resistant species, but prevalence and severity of IOD pale in comparison to what is exhibited by SRs and BRs. Taken together, these results do not support an association between serum ORP and IOD in the rhinoceros. However, there are many ways to evaluate ORP and many types of ROS to measure, and this study focused only on a single assay with tight association to iron and the Fenton reaction in serum. In contrast, a potential positive relationship between serum oxidative stress markers and IOD in BRs has been suggested (Pouillevet *et al*., 2020), but measures of serum oxidative stress markers capture by-products of intracellular redox activity unrelated to serum ORP. Studies using varied approaches to assess oxidative activity within organisms are likely to yield different results, all of which are valuable in deciphering the physiological pathways potentially impacted by IOD.

In contrast to the sex impact, rhinoceros age at time of sampling was not a significant covariate, nor was it significantly correlated with sample ORP, and results did not change when SR data were removed from the analysis. Because serum ORP appears high in three rhinoceros species compared with normal, healthy levels recorded for humans using the same methodology (Esposito *et al*., 2003), it is possible that age-related shifts in ORP were masked by the disproportionately high ORP unrelated to ageing changes. However, the theory that increasing oxidative damage is associated with ageing and mortality (Harman, 1956) has been debated in recent years, and it is now largely believed that the physiological pathways of ROS and antioxidant production and function are extremely complex with both positive and negative consequences on health depending on the circumstances (Egea *et al*., 2017; Kirkwood and Kowald, 2012; Santos *et al*., 2018). Therefore, although our study revealed higher than expected ORP in three rhinoceros species tested with this specific LPI assay, it would be premature to speculate on how these oxidation rates relate to rhinoceros health.

Because it has already been shown that serum ferritin concentration is not an accurate indicator of IOD in rhinoceroses (Ball and Mueller, 2011; Roth *et al*., 2017; Wojtusik and Roth, 2018), it was not surprising that BR samples in all ferritin categories (low, moderate and high) tested positive for LPI. Ferritin is an acute phase protein that increases with inflammation (Lee and Means, 1995; Wang *et al*., 2010), and some inflammatory markers in managed BR appear elevated compared with those of wild rhinoceroses (Schook *et al*., 2015). Because ROS is also known to increase in association with inflammatory diseases such as cancer (Prasad *et al*., 2017), rheumatoid arthritis (Khojah *et al*., 2016), diabetes and obesity (Matsuda and Shimomura, 2013), it seemed likely that ROS and serum ferritin would be correlated within BRs, and our data do support this positive relationship, although with $r = 0.43$, it was not a particularly strong association.

Although rhinoceros serum iron concentrations and liver iron load are not tightly linked, there are several reports indicating that BR and SR serum iron tends to be higher than that of WRs and GOH rhinoceros (Dierenfeld *et al*.,

2005). However, there is considerable overlap in the range of values among species. Serum iron data reported herein support these previous reports. Given the range in serum iron among individuals, one would anticipate that samples containing higher iron concentrations would be more likely to test positive for LPI. Esposito *et al.* (2003) demonstrated a positive linear relationship between DHR oxidation rate and iron concentrations in serum from thalassemic patients and also reported that LPI became particularly prominent in samples with high iron load. In contrast, our data indicate that there is no relationship between DHR oxidation rate and total serum iron in the rhinoceros. Furthermore, rhinoceros serum iron was not significantly higher in LPI-positive versus -negative samples, although there was a trend towards significance. The latter result is in accordance with data from thalassemic patients in which the presence of LPI was not uncommon in samples with relatively low iron loads (Esposito *et al.*, 2003). It was postulated that the transferrin–iron binding properties may be aberrant in such samples. Because rhinoceros serum transferrin saturation has proven challenging to measure, even in commercial laboratories, it is possible that the transferrin–iron binding properties of the rhinoceros differ from those in domestic species, but delving further into the theory was well beyond the scope of this study.

To our knowledge, this is the first report of measuring LPI in serum of a wildlife species. Although the assay was consistently reliable yielding quality data, our results did not support a strong connection between LPI-positive samples and IOD in rhinoceroses. Another by-product of this study was the data generated on redox activity in rhinoceros serum, which revealed a significant sex bias not previously reported in the few existing studies on the topic (Paglia *et al.*, 1996; Pouillevet *et al.*, 2020). Together, these new findings drive home the importance of comparative studies within the Rhinocerotidae. Assuming rhinoceros physiology and normative values, patterns and associations are similar to those for humans, or for laboratory, domestic or agricultural species, has led to erroneous conclusions. The theory that unique red blood cells (RBC) characteristics were linked to IOD in BRs dissipated as other rhinoceros species and wild rhinoceroses were studied and found to be comparable (Paglia *et al.*, 1996). Similarly, in this study, the BR high ORP and positive LPI results revealed in Study 1 lost their novelty after data for WR and GOH rhinoceros were generated in Study 2. Furthermore, it is becoming apparent that the aetiology of IOD among rhinoceros species differs. Despite the two most susceptible species' similar propensity for organ tissue hemosiderosis accumulation (Paglia and Tsu, 2012) and reduced gut microbiome diversity (Roth *et al.*, 2019b), serum, genetic and metabolic biomarker disparity between BR and SRs suggest significant aetiological divergence or disassociation with IOD (Beutler *et al.*, 2001; Roth *et al.*, 2017; Salyer, 2017; Wojtusik and Roth, 2018; Roth *et al.*, 2019b). Although much about IOD in rhinoceroses remains elusive, the pursuit of novel scientific avenues and methodologies in recent years is shedding light on valuable pieces of the complex puzzle that may eventually coalesce and provide clarity.

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Author contributions

T.L.R. conceived the study, acquired the samples, performed the experiments, analysed the data, interpreted the data and drafted the manuscript. J.W. provided ferritin data, helped with study design, data interpretation and manuscript preparation. M.P. was responsible for the R statistical analyses/interpretation and preparation of relevant portions of the manuscript.

Data availability statement

The data that support the findings of this study are available from the corresponding author (T.L.R.) upon reasonable request. All information associated with individual rhino identification will be removed in accordance with our commitment of anonymity to participating facilities.

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