A Retrospective Study on the Correlation Between Serum 25(OH)D and Plasma Phosphate in Black Rhinoceroses (*Diceros bicornis michaeli*) at Rotterdam Zoo (2020 - 2024)

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Introduction

The population of free-roaming black rhinoceroses (*Diceros bicornis*) has seen a dramatic decline of 94% between 1960 and 2018 (IUCN, 2020), resulting in its designation as critically endangered by the International Union for Conservation of Nature (IUCN). Multiple efforts for conservation have been made, including preserving this species in zoos across the world. Relocating black rhinoceroses from their natural habitat can pose health risks to these animals. A study conducted by Molenaar *et al.* (2008) has found significantly lower values of inorganic phosphorus in captive rhinoceroses in the United Kingdom compared to their wild counterparts. Additionally, hypophosphatemia has been reported multiple times in captive ill black rhinoceroses (Gillespie *et al.*, 1990; Murray *et al.*, 2000), and it has been suggested that it could play a role in the pathophysiology in multiple diseases such as haemolytic anaemia (Paglia, 2007), iron overload disorder (Schook *et al.*, 2015), ulcerative stomatitis (Gillespie *et al.*, 1990) and superficial necrolytic dermatitis (Dennis *et al.*, 2007). Therefore, gaining knowledge in the physiology of the phosphate metabolism in this species could enhance the health and the welfare of captive animals and aid the conservation of this endangered animal outside of its natural habitat.

It is known that phosphate is regulated by vitamin D in multiple species, including humans, dogs, horses, alpaca's and guinea pigs (Fukumoto, 2014; Harmeyer & Schlumbohm, 2004; Hurst *et al.*, 2020; Simboli-Campbell & Jones, 1991; Van Saun *et al.*, 1996). In many animals, vitamin D is present in two forms, namely vitamin D_2 and D_3, both obtained via different routes (Hurst *et al.*, 2020). Vitamin D_2 is consumed via a plant-based diet, after the synthesization of ergosterol in fungi and yeast by ultraviolet B (UVB) radiation. In humans and some animals, vitamin D_3 is synthesized in the skin when UVB radiation converts 7-dehydrocholesterol to vitamin D_3. After entering the body, the fat soluble vitamin D_2 and D_3 are transported to the liver, predominantly bound to vitamin D binding protein. Vitamin D_2 and D_3 are first converted to 25-hydroxyvitamin-D_2/D_3 (25(OH)D_2/D_3) through hydroxylation at C25, and then further hydroxylated at C1α to form the most biologically active form, 1α,25-dihydroxyvitamin-D_2/D_3 (1,25(OH)2D_2/D_3) (Bikle, 2014; Corbee, 2020; Hurst et al., 2020).

1,25$(OH)_2D_2/D_3$ is important in the regulation of extracellular phosphate homeostasis. This process is tightly regulated, as phosphate plays a key role in cellular metabolism and structural function (being a part of the cell membrane and nucleic acids), generating ATP for cellular metabolism, phosphorylation of enzymes and maintenance of acid-base in urine and blood (Amanzadeh & Reilly, 2006; Jacquillet & Unwin, 2019). Phosphate is primarily stored in bones and teeth as hydroxyapatite, with less than 1% present in extracellular fluids in humans (Penido & Alon, 2012). Besides 1,25$(OH)_2D_2/D_3$, extracellular phosphate homeostasis is also regulated by other endocrine factors including parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF-23).

In humans, there are roughly three main ways in which 1,25$(OH)_2D_2/D_3$ regulates the phosphate homeostasis (Jacquillet & Unwin, 2019). First, 1,25$(OH)_2D_2/D_3$ stimulates the absorption of intestinal phosphate by stimulating intestinal phosphate transporters.

Moreover, osteoclast and osteoblast cells in bone are stimulated by $1,25(OH)_2D_2/D_3$ to increase FGF-23 production. Subsequently FGF-23 inhibits Na-P transporters in the proximal tubules in order to decrease renal phosphate reabsorption (resulting in a decrease in extracellular phosphate concentration) and in the proximal tubules it also inhibits renal $1,25(OH)_2D_2/D_3$ production. And lastly, $1,25(OH)_2D_2/D_3$ inhibits the parathyroid gland so it decreases the synthesis of PTH (indirectly inhibiting bone resorption and increasing renal phosphate reabsorption, both increasing extracellular phosphate). Furthermore, $1,25(OH)_2D_2/D_3$ regulates itself, as high levels of $1,25(OH)_2D_2/D_3$ inhibit the proximal tubules from producing more vitamin D (Fukumoto, 2014; Jacquillet & Unwin, 2019). In summary, a deficiency of $1,25(OH)_2D_2/D_3$ reduces the uptake of intestinal phosphate and it increases PTH levels which cause increased phosphate excretion via the urine, which can both cause hypophosphatemia (Sarathi *et al.*, 2024).

Two recent studies suggested that vitamin D levels in black rhinoceroses seem to associate with sunlight exposure and UV radiation, resulting in low serum 25(OH)D levels in captive individuals in the northern hemisphere, particularly during winter months (Bruins-van Sonsbeek & Corbee, 2024; Olds *et al.*, 2018). However, it remains unclear whether a correlation exists between vitamin D and plasma phosphate levels in black rhinoceroses or whether vitamin D deficiency contributes to hypophosphatemia. This study aims to investigate this potential correlation by analysing serum 25(OH)D and plasma phosphate samples from five Eastern black rhinoceroses (*Diceros bicornis michaeli*) at Rotterdam Zoo, the Netherlands, incorporating historical data from 2020 to 2024. To assess vitamin D status, this study measures serum 25(OH)D, as it is a reliable indicator of vitamin D status due to its relative abundance in circulation, ease of analysis, stability, and half-life (Hurst *et al.*, 2020). Additionally, seasonal changes in plasma phosphate levels will be evaluated in relation to UV radiation and compared to serum 25(OH)D. Furthermore, longitudinal changes will be examined to evaluate the impact of environmental changes during the course of this study. Understanding the relationship between serum 25(OH)D and plasma phosphate is essential for refining dietary strategies, enhancing environmental enrichment, and optimizing UV exposure protocols, ultimately improving the health and welfare of captive black rhinoceroses and contributing to global conservation efforts.

Table 1. Detailed information about participating animals and number of collected samples per animal.

Individual	Sex	Date of birth	Age at sample collection	*n* 25(OH)D samples	*n* phosphate samples
1	m	8-11-2001	18 - 20	19	27
2	f	10-12-2011	8 - 12	55	61
3	f	23-12-2017	2 - 4	22	31
4	m	8-11-2020	0 - 4	26	42
5	m	28-04-2019	4 - 5	12	12

Materials and methods

Study population

This study is based on retrospective data collected from five Eastern black rhinoceroses (*Diceros bicornis michaeli*) housed at Rotterdam Zoo between 2020 and 2024. The study population included three males (aged 0 to 20 years) and two females (aged 2 to 12 years), with individual animal details presented in Table 1. The rhinoceroses had full outdoor access when nighttime temperatures were above 12 °C. On colder days, when outdoor temperatures fell below 5 °C, access to outdoor enclosures was restricted to approximately 10:00 to 15:30 CET. Their diet consisted primarily of roughage and pellets. On average, each rhinoceros received approximately 2 kg of pellets per day, containing 8 g/kg phosphate and 1500 IU/kg of vitamin D_3. Additionally, their daily roughage intake included 20 kg of rose, 15 kg of alfalfa (with phosphate content fluctuating; the most recent analysis indicated 2.1 g/kg), and two bunches of willow.

Vitamin D and phosphate analysis

The blood samples and analysis in this study were originally collected as part of routine health monitoring, and permission was granted by the zoo for the use of the data. Blood samples were drawn from the medial radial vein or interdigital vein using a Covetrus® 21-gauge winged needle and collected in a SST II Advance vacutainer for serum 25(OH)D and a Lithium Heparin vacutainer for plasma phosphate analysis. After collection, serum and plasma samples were centrifuged at 3000 RPM for 10 minutes using a Hettich Rotofix 32A® centrifuge. Total serum 25(OH)D was analysed using a VIDAS® enzyme-linked fluorescent assay. The VIDAS® 25(OH)D Vitamin Total D assay employs an immunoenzymatic method to measure total vitamin D, with a detection range of 20.3 - 315 nmol/L. Plasma phosphate was measured using the Skyla® Solution Immunoassay Analyzer. Plasma phosphate concentration was determined using colorimetry, involving a series of enzymatic reactions, with absorbance measured at a light wavelength of 340 nm. The detection range for plasma phosphate was 0.13 - 5.81 mmol/L.

Data collection

Serum 25(OH)D and plasma phosphate data were obtained from the animals' health records in ZIMS 360. The routine sampling was performed on weekly basis. Sample collection did not follow a fixed schedule and performed voluntarily, some months had no available samples, while others had multiple measurements for either 25(OH)D, phosphate or both. When multiple samples were available for a given month, priority was given to those where both 25(OH)D and phosphate were collected simultaneously. If measurements were taken in the same month but not concurrently, they were still analysed as a pair for that month. In cases where multiple concurrent samples were available within the same month, the average of both measurements was used for analysis. This approach was applied to individual 2 in December 2021 (n = 2), October 2022 (n = 2), and November 2022 (n = 3), as well as to individual 4 in April 2023 (n = 2), where n represents the number of concurrent samples averaged.

In total, 307 unique blood samples were analysed, comprising 134 serum 25(OH)D measurements and 173 plasma phosphate measurements. Two samples were excluded due to abnormally high phosphate concentrations, which were accompanied by multiple distorted biochemical values. These excluded samples were collected from Rhinoceros 1 in September 2020, with a phosphate concentration of 3.50 mmol/L, and from Rhinoceros 2 in February 2023, with a phosphate concentration of 3.97 mmol/L. In the latter case, two paired 25(OH)D and phosphate samples were available, and the pair with the normal phosphate value was retained for analysis. After excluding one phosphate sample and averaging multiple samples collected within a single month, the final dataset comprised 128 serum 25(OH)D samples, 167 plasma phosphate samples, and a total of 126 paired 25(OH)D-phosphate measurements.

UV index

The UV index is a scale that measures the strength of ultraviolet (UV) radiation from the sun at a specific location and time, commonly used as an indicator of sunburn risk in humans (KNMI, 2025). The scale ranges from 0 to 11+, with values of 0 - 2 classified as low, 3 - 5 as moderate, 6 - 7 as high, 8 - 10 as very high, and 11+ as extreme radiation levels (CAMS, 2025). This study used historical UV data from de Bilt, Netherlands, between 2020 and 2024, obtained from the Tropospheric Emission Monitoring Internet Service, operated by the European Space Agency (TEMIS, 2025). Monthly averages were calculated and visualized using Microsoft Excel®.

To facilitate seasonal comparisons, the study period was divided into four categories based on UV index values. Winter (December - February) included months with a UV index < 1, while summer (June - August) encompassed months with the highest UV index > 5. Spring (March - May) and autumn (September - November) were classified as transitional periods with UV index levels roughly between 1 - 5.

Statistical analysis

To evaluate the correlation between serum 25(OH)D and plasma phosphate levels, a linear regression analysis was performed in R studio (version 2024.12.0), with 25(OH)D as the independent variable and phosphate as the dependent variable. Preliminary visualization suggested clustering by individual, indicating that measurements were not independent. To account for individual-specific effects, a random effects regression model was applied instead of a standard linear regression.

Model assumptions were evaluated before interpreting the regression results. Linearity was initially assessed through visual inspection and further confirmed using a 25(OH)D ~ residuals plot. Homoscedasticity was evaluated by plotting predicted phosphate values ~ residuals, and normality was examined using a Q-Q plot and a histogram of the residuals. Since the phosphate data were normally distributed but the 25(OH)D data were not, a logarithmic transformation was applied to 25(OH)D before regression analysis.

To compare seasonal and yearly variations in 25(OH)D and phosphate levels, data were grouped by season and year based on the collection date. Boxplots were generated for

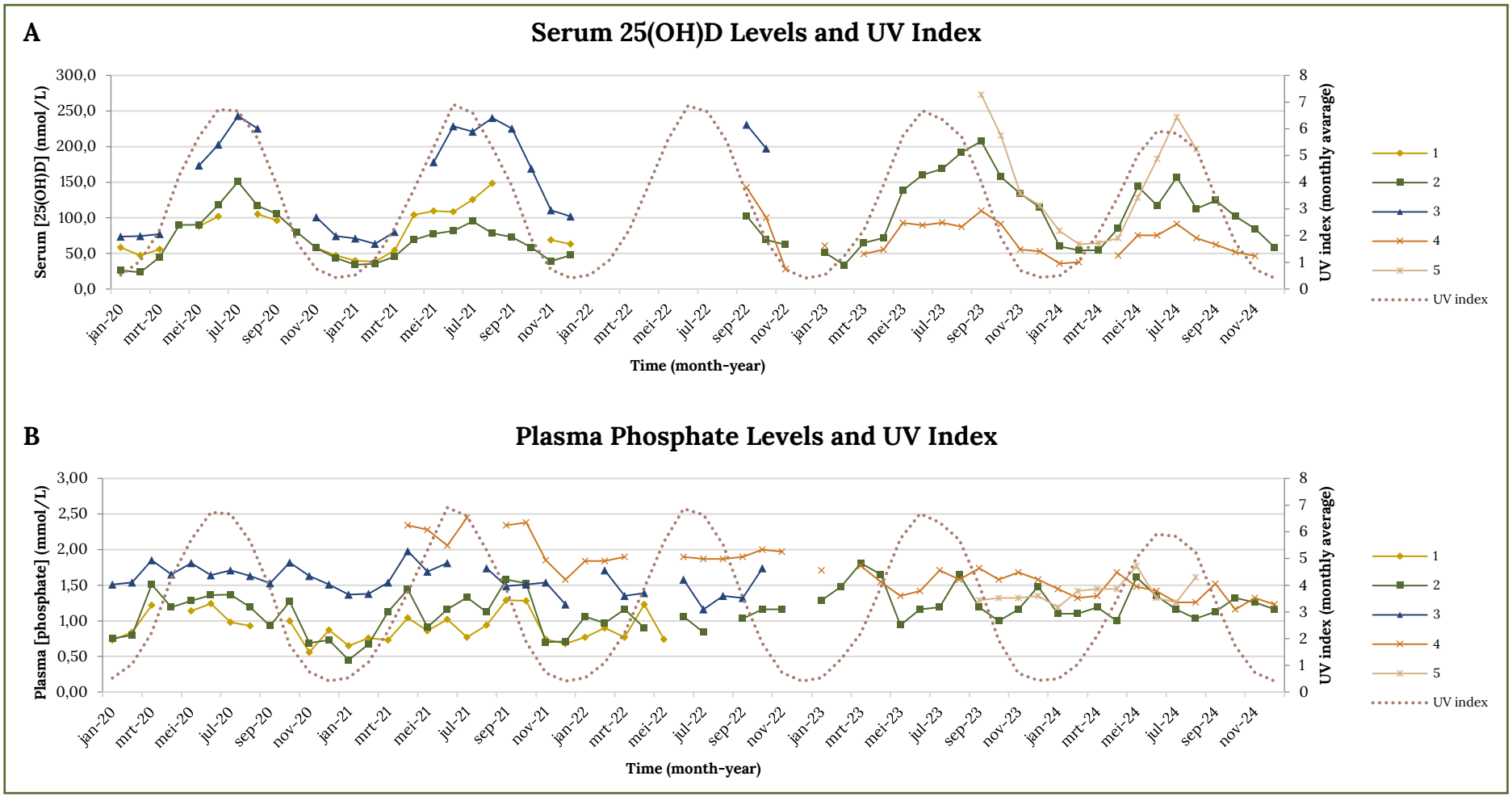

Figure 1. Monthly average of the UV index and the concentrations of (**A**) serum 25(OH)D in nmol/L and (**B**) plasma phosphate in mmol/L from 2020 to 2024. The numbers in the legend correspond to the individuals outlined in Table 1.

both seasonal and yearly datasets to visualize potential trends. Since both log-transformed 25(OH)D and phosphate data were normally distributed, a one-way ANOVA was conducted to assess statistical differences across seasons and years. Tukey's range test was performed to compare pairs of seasons and years.

The assumptions of ANOVA were evaluated using Levene's test for homogeneity of variances and Q-Q plots and scale-location plots for normality. All datasets met these assumptions, except for yearly phosphate levels, which violated the assumption of homogeneity. Consequently, a Welch's ANOVA was performed for yearly phosphate data to account for unequal variances. A significance level of $p < 0.05$ was used for all statistical tests.

Results

Figure 1 presents serum 25(OH)D and plasma phosphate concentrations per rhinoceros over time, alongside the average monthly UV index from 2020 to 2024. Figure 2 displays a scatterplot of the random effects regression model, illustrating the relationship between log-transformed serum 25(OH)D levels (ln(nmol/L)) and plasma phosphate levels (mmol/L). The data points in both figures are color-coded by individual rhinoceroses. The regression line follows the equation $y = 0.150x + 0.635$. The coefficient has a 95% confidence interval of 0.140 - 0.541, and the intercept has a 95% confidence interval of 0.193 - 1.077.

Figure 3 shows boxplots of the seasonal distribution of serum 25(OH)D and plasma phosphate concentrations, with details provided in Table 2. A summary of the p - values for seasonal comparisons is presented in Table 3. Significant differences were observed between seasonal serum 25(OH)D levels for all seasons, except spring and autumn. For plasma phosphate, significant differences were only observed when comparing winter to the other seasons, with winter values being lower than the rest of the year.

Figure 4 presents boxplots of serum 25(OH)D and plasma phosphate concentrations per year, with detailed information provided in Table 4. No statistically significant differences were found when comparing yearly distributions of either parameter.

Discussion

Study limitations

This study was conducted on five black rhinoceroses housed in a single zoological facility. While this controlled setting ensured consistent sampling conditions, it limits the generalizability of the findings. Comparing these results with data from rhinoceroses in other zoos or geographic regions, particularly at varying latitudes where seasonal changes in sunlight hours and UV index intensity differ, could provide further insight into environmental influences on vitamin D metabolism and its effect on phosphate levels. Generalizability could also be improved by including animals from additional zoological institutions with differing diets, enclosure types, group compositions, and other environmental conditions that may influence physiological parameters.

The relatively small sample size further limits the statistical power of the study. A larger group of animals would increase the ability to detect subtle patterns in serum 25(OH)D and plasma phosphate concentrations, thereby improving the robustness of the statistical analyses. Additionally, not all five rhinoceroses were sampled consistently throughout the study period, as individuals joined or left the group at different times. This variation in group composition complicates the interpretation of individual trends over time and challenges population-level analyses.

Furthermore, because the data were collected retrospectively, environmental changes over the study period, such as alterations in diet, husbandry, and housing, were not systematically documented. This complicates the interpretation of their potential impact on the observed patterns. The study design also did not include a detailed dietary analysis or a summary of dietary changes over the course of the study period. Given the established role of diet in regulating both phosphate levels and vitamin D metabolism, future research incorporating comprehensive dietary assessments would provide valuable additional insights.

Correlation between 25(OH)D and phosphate

The scatterplot in Figure 2 highlights individual differences among the rhinoceroses, with rhinoceroses 1 and 2 displaying relatively lower phosphate levels than rhinoceros 3 and 4. A random effects regression model was applied to account for these differences. Table 2

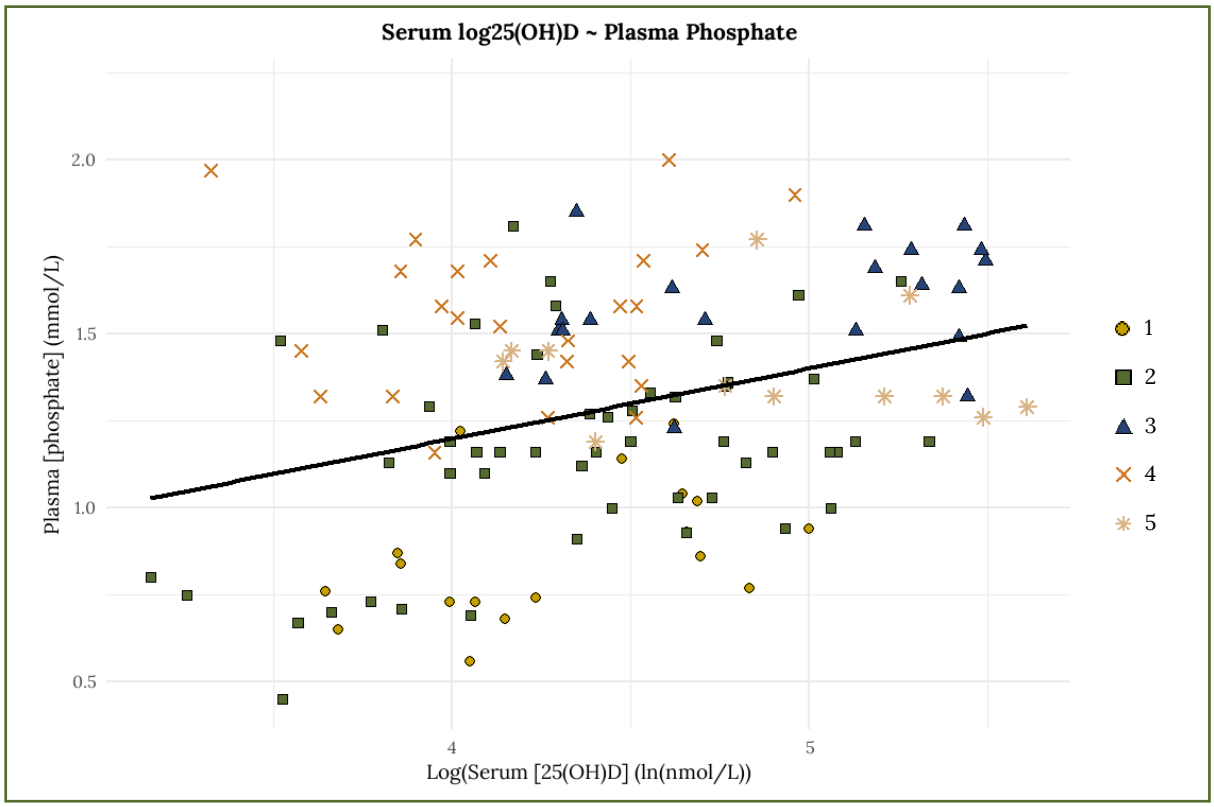

Figure 2. Scatterplot of serum log(25(OH)D) concentration in ln(nmol/L) as the independent variable and plasma phosphate concentration in mmol/L as the dependent variable, with a regression line from a random effects regression model. The points are color-coded by individual, and the numbers in the legend correspond to the individuals outlined in Table 1.

presents information on the serum 25(OH)D and plasma phosphate data analysed in this study. On a logarithmic scale, the median serum 25(OH)D concentration is approximately 4.44 ln(nmol/L), with a interquartile range (IQR) from 4.07 to 4.62 ln(nmol/L).

The statistically significant positive correlation between log-transformed 25(OH)D and phosphate suggests a potential biological link between vitamin D metabolism and phosphate homeostasis in black rhinoceroses. Clinically, the logarithmic scale implies that small increases in 25(OH)D at lower levels may have a greater impact on phosphate concentrations than similar increases at higher levels, suggesting a possible threshold effect in vitamin D metabolism, where lower levels are more critical for regulating phosphate homeostasis.

The scatterplot shows that most data points fall between a concentration of 0.9 and 1.9 mmol/L plasma phosphate. However, some inconsistencies are present, with certain samples showing high phosphate levels despite low 25(OH)D values and vice versa. Additionally, a smaller cluster appears for rhinoceroses 1 and 2, with serum 25(OH)D below 4.3 ln(nmol/L) and phosphate below 0.9 mmol/L. This pattern may suggest that rhinoceroses 1 and 2 might not be able to maintain higher phosphate levels when their 25(OH)D is low. Interestingly, Rhino 2 exhibits some exceptions, maintaining stable phosphate levels despite low 25(OH)D. A comparison between Figures 1 and 2 indicates that these exceptions occurred at the onset of 2023 when rhinoceros 2 maintained a stable plasma phosphate concentration throughout the year.

Seasonal trends in 25(OH)D and phosphate levels

Seasonal fluctuations in serum 25(OH)D levels were evident, as shown in Figure 1A. Concentrations followed a pattern similar to the UV index, with 25(OH)D levels increasing during the summer months and decreasing in winter, trailing slightly behind the UV index trend. This seasonal pattern aligns with previous studies demonstrating a correlation between vitamin D levels and UVB exposure in black rhinoceroses (Bruins-van Sonsbeek & Corbee, 2024; Olds *et al.*, 2018).

Remarkably, there were considerable individual differences among the rhinoceroses. During the first half of the study (2020 - 2021), rhinoceros 3 maintained higher serum 25(OH)D levels both in summer and winter compared to rhinoceroses 1 and 2. During the second half of the study (2023 - 2024), rhinoceroses 2 and 5 reached considerably higher 25(OH)D values than rhinoceros 4, highlighting inter-individual variability even under similar environmental conditions. Notably, rhinoceros 2, who was followed throughout the entire study period, achieved higher summer vitamin D levels in 2023 and 2024 than in 2020 and 2021.

Phosphate levels were more stable than 25(OH)D levels, with only mild seasonal changes and a weaker correlation with UV index (Figure 1B). However, from January 2020 to November 2022, plasma phosphate levels tended to decline during winter, particularly when the UV index fell below approximately 2. In contrast, from mid-2023 onward, plasma phosphate levels in rhinoceroses 2, 4, and 5 remained remarkably stable across all seasons.

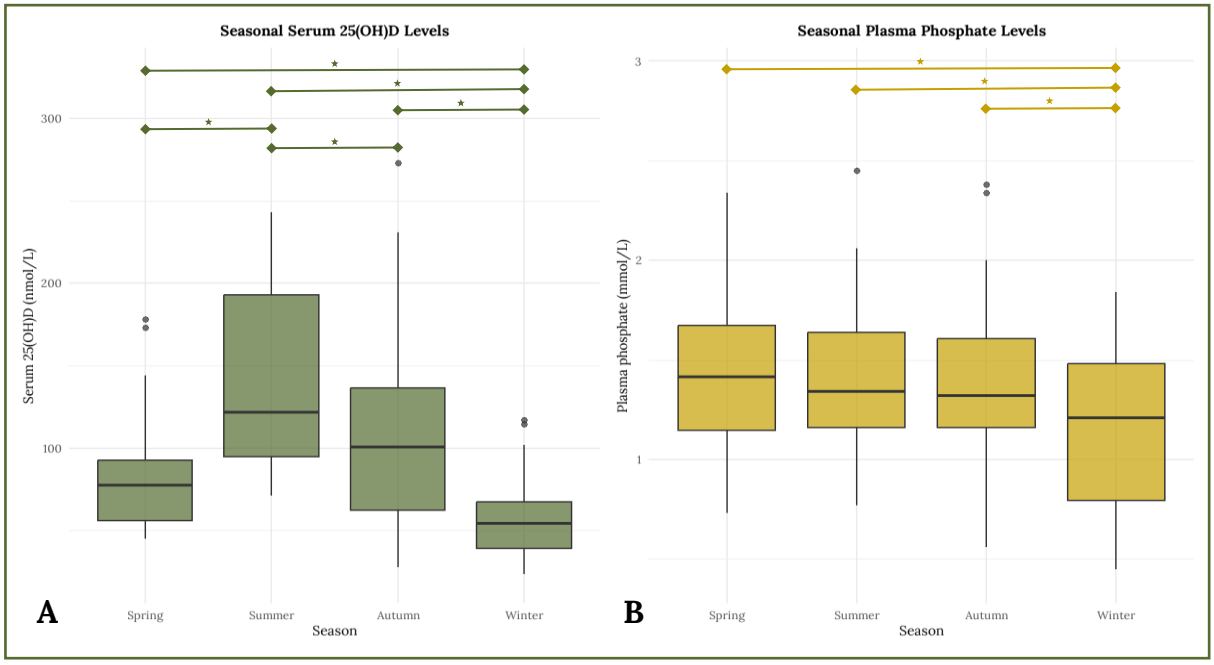

Figure 3. Seasonal distribution of (**A**) serum 25(OH)D concentrations in nmol/L and (**B**) plasma phosphate concentrations in mmol/L. Each boxplot shows the median, interquartile range, and potential outliers for all collected data points per season. The bar with ★ indicate statistical significant differences between two seasons.

Table 2. Seasonal summary of serum 25(OH)D and plasma phosphate concentrations.

Samples	Mean (SD)	Median (IQR)	Median per Season (IQR)			
			Dec - Feb UV Index < 1	*Mar - May* UV Index 1 - 5	*Jun - Aug* UV Index > 5	*Sep - Nov* UV Index 1 - 5
Serum 25(OH)D	101 (57.3)	85.0 (58.3 - 101)	54.3 n = 31 (39.0 - 67.3)	77.3 n = 29 (56.0 - 92.8)	122 n = 32 (94.6 - 193)	101 n = 36 (62.5 - 136)
Plasma phosphate	1.34 (0.393)	1.32 (1.10 - 1.60)	1.21 n = 40 (0.793 - 1.48)	1.42 n = 42 (1.14 - 1.67)	1.34 n = 42 (1.16 - 1.64)	1.32 n = 43 (1.16 - 1.60)

This table presents the mean, standard deviation (SD), median, and interquartile range (IQR) for all samples of serum 25(OH)D (nmol/L) and plasma phosphate (mmol/L). Additionally, it provides the median values and sample sizes with corresponding IQRs for both serum 25(OH)D and plasma phosphate (mmol/L) per season based on the UV index.

Table 3. Results of pairwise seasonal comparisons in serum 25(OH)D and plasma phosphate levels.

Seasonal Pairs	p - Values 25(OH)D	p - Values Phosphate
Spring - Autumn	0.269	0.998
Summer - Autumn	**0.015**	0.999
Winter - Autumn	**0.000**	**0.040**
Summer - Spring	**0.000**	0.998
Winter - Spring	**0.003**	**0.027**
Winter - Summer	**0.000**	**0.044**

Seasonal differences were evaluated using post-hoc Tukey's range tests following one-way ANOVA. The table summarizes the p - values for each seasonal comparison, with statistically significant differences highlighted in bold.

Interestingly, this stabilization in phosphate levels in rhinoceros 2 coincided with the observed changes in its serum 25(OH)D levels. This suggests a possible environmental factor, such as diet, influencing both vitamin D and phosphate homeostasis. Further investigation into dietary modifications or management changes during this period could provide valuable insight.

Notably, rhinoceros 4 exhibited relatively high phosphate levels between March 2021 and December 2022 compared to the other individuals during that period. This may be attributable to age-related factors, as rhinoceros 4 was born in November 2020 and was only four months old when the first blood samples were collected. Research in multiple species such as humans, dogs, cattle and goats show that younger animals typically have higher phosphate levels due to increased bone growth and metabolic demands, which may explain this observation (Cirillo *et al.*, 2010; Doornenbal *et al.*, 1985; Russo & Nash, 1980; Zaeemi *et al.*, 2016). However, rhinoceros 3, who was between two and four years old during the study, had phosphate levels that fell within the expected range, suggesting that phosphate levels may decrease approximately beyond the first year of life or that factors such as diet, metabolism, or individual variation may also contribute.

The seasonal trends illustrated in Figure 1 are further supported by the boxplots in Figure 3. Figure 3A shows statistically significant variation in serum 25(OH)D levels between all seasons, except between spring and autumn. Plasma phosphate levels however (Figure 3B), were only statistically lower in winter compared to other seasons. Interestingly, the median and the upper whisker for spring are higher than those for summer and autumn, despite following winter, the season with the lowest UV index. While one might expect spring levels to be lower than summer and autumn, this difference may not be statistically significant and could reflect inter-individual variation.

Longitudinal Changes and Dietary Impact

To assess long-term trends in serum 25(OH)D and plasma phosphate levels, yearly boxplots were generated (Figure 4), providing insight into annual variation. The details of the yearly boxplots can be found in Table 3. While no statistically significant differences were observed between years for neither 25(OH)D nor phosphate levels, several noteworthy patterns emerged. Serum 25(OH)D levels remained relatively stable throughout the study period, with 2020, 2021, and 2024 showing the most similar distributions. In contrast, 2022 exhibited the highest median 25(OH)D concentration, though this should be interpreted with caution due to the low number of samples collected that year (n = 8). The most pronounced variation occurred in 2023, which had the widest range of 25(OH)D values. This increased variability can be attributed to individual differences, such as rhinoceros 2 reaching higher summer 25(OH)D levels compared to 2020 and 2021. Additionally, rhinoceros 5, which joined the group in 2023, exhibited relatively high summer 25(OH)D levels, contributing to the observed variability.

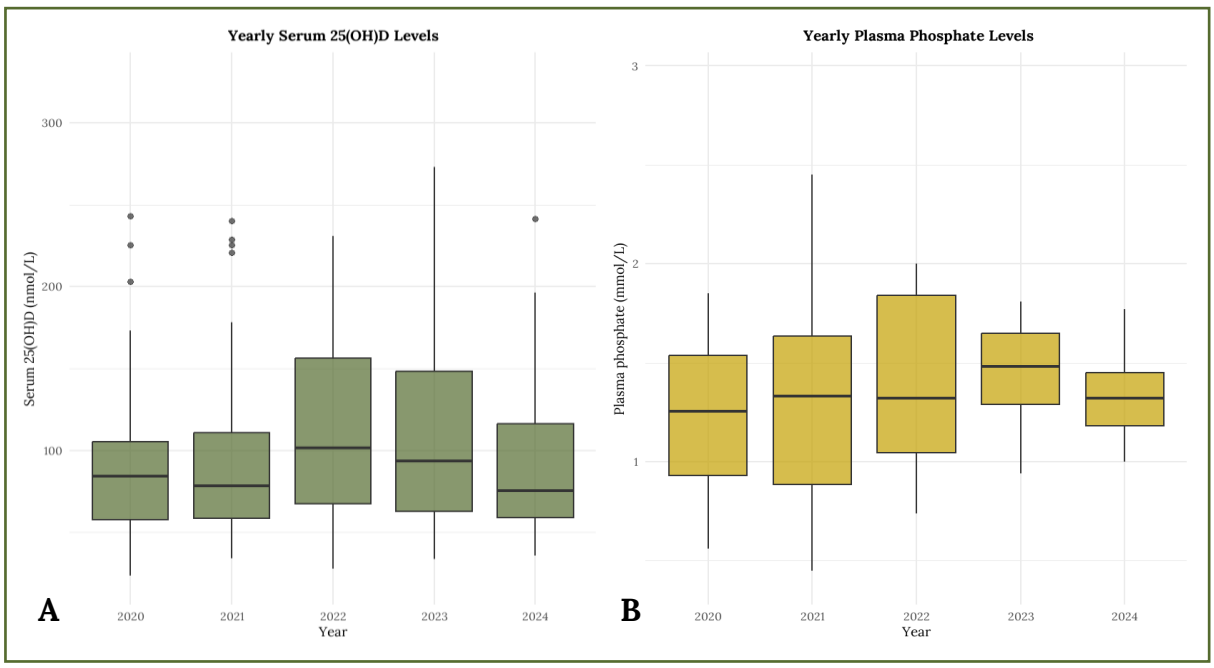

Figure 4. Yearly distribution of **(A)** serum 25(OH)D concentrations in nmol/L and **(B)** plasma phosphate concentrations in mmol/L from 2020 to 2024. Each boxplot shows the median, interquartile range, and potential outliers for all collected data points per year.

Table 4. Yearly summary of serum 25(OH)D and plasma phosphate concentrations.

Samples	Mean (SD)	Median (IQR)	Median per Year (IQR)				
			2020	2021	2022	2023	2024
Serum 25(OH)D	101 (57.3)	85.0 (58.3 - 101)	84.2 n = 30 (57.5 - 105)	78.5 n = 33 (58.3 - 111)	102 n = 8 (67.4 - 156)	93.3 n = 27 (62.8 - 148)	75.4 n = 30 (58.8 - 116)
Plasma phosphate	1.34 (0.393)	1.32 (1.10 - 1.60)	1.25 n = 34 (0.93 - 1.54)	1.33 n = 43 (0.89 - 1.64)	1.32 n = 31 (1.04 - 1.84)	1.48 n = 27 (1.29 - 1.65)	1.32 n = 32 (1.18 - 1.45)

This table presents the mean, standard deviation (SD), median, and interquartile range (IQR) for all samples of serum 25(OH)D (nmol/L) and plasma phosphate (mmol/L). Additionally, it provides the median values and sample sizes with corresponding IQRs for both serum 25(OH)D and plasma phosphate (mmol/L) per year.

Figure 4B illustrates plasma phosphate levels decreasing in overall range in recent years, with boxplots narrowing in 2023 and 2024. Although no statistically significant differences were observed, the reduced spread suggests a trend toward more consistent phosphate levels across the rhinoceroses in the study. One potential contributing factor is the absence of a clear seasonal decline in phosphate levels during winter in the later years, which may have reduced overall variation. Additionally, this trend could be related to a more standardized diet, leading to less fluctuation in phosphate intake, or possibly other generalized environmental factors that resulted in more uniform phosphate levels across individuals. This decrease in variability highlights the need for further investigation to determine whether specific environmental, physiological, or dietary factors contributed to the observed stabilization of phosphate levels in recent years. It is also worth noting that the 2023 boxplot displayed a higher median, likely due to the fact that phosphate measurements for most of that year were derived from only two rhinoceroses, one of whom was Rhinoceros 4, who exhibited relatively high plasma phosphate levels throughout the study period.

Looking back to 2021, phosphate levels displayed the widest interquartile range, likely influenced by the inclusion of rhinoceros 4, as mentioned earlier. This broad range persisted into 2022, although with some differences: while the median remained similar, the upper quartile was higher in 2022. These shifts may reflect rhinoceros 4's consistently higher phosphate levels, as well as the departure of rhinoceros 1, who had relatively lower phosphate concentrations.

Additional factors in vitamin D metabolism

While this study primarily focused on serum 25(OH)D and plasma phosphate, other key regulators, such as calcium, PTH, and FGF-23, also play crucial roles in vitamin D metabolism and phosphate homeostasis. Although 25(OH)D is commonly used as a marker for assessing vitamin D status, it is not the biologically active form involved in regulating phosphate levels. Instead the hormonally active metabolites $1,25(OH)_2D_2/D_3$ modulate calcium and phosphate absorption. Therefore, measuring circulating levels of $1,25(OH)_2D_2/D_3$, alongside phosphate concentrations and other key regulators, in future studies could provide a more complete understanding of the functional relationship between vitamin D activity and phosphate metabolism in black rhinoceroses.

Interestingly, the rhinoceroses in this study did not exhibit apparent hypophosphatemia, as their plasma phosphate concentrations remained within or near reference ranges. Comparing these phosphate study results to previous findings; Schook *et al.* (2015) reported a median serum phosphate concentration of 1.45 mmol/L (95% confidence intervals (CI): 0.74 - 2.10 mmol/L, n = 86) in free-ranging black rhinoceroses and 1.58 mmol/L (CI: 0.45 - 5.20 mmol/L, n = 117) in zoo-managed individuals, using the Idexx® Vet Test 8008 chemistry analyser. Similarly, Molenaar *et al.* (2008) found mean serum inorganic phosphate concentrations of 1.20 mmol/L (CI: 0.57 - 1.82 mmol/L, n = 27) in free-ranging and 0.73 mmol/L (CI: 0.61 - 1.98 mmol/L, n = 17) in zoo-housed rhinoceroses, measured using the ILAB® 600 chemistry analyser after serum samples were stored at -20 °C and thawed. Both studies used serum samples to determine phosphate levels, in contrast to plasma phosphate samples analysed in this study. Carey *et al.* (2016) compared 101 paired human serum and plasma samples and concluded that the two sample types can generally be used interchangeably for phosphate analysis, although serum may yield slightly higher values due to phosphate release during the clotting process, with differences up to -0.065 mmol/L. Furthermore, while the impact of sample storage was noted in Molenaar *et al.*, similar research on rat serum (Kale *et al.*, 2012) demonstrated no clinically relevant changes in phosphate levels after one freeze-thaw cycle at -10 to -20 °C, supporting the reliability of the stored serum samples in Molenaar *et al.*'s study.

While captive black rhinoceroses typically receive higher levels of dietary phosphate than their free-ranging counterparts, free-ranging individuals do not appear to suffer from clinical signs of phosphate deficiency (Clauss *et al.*, 2007). This suggests that hypophosphatemia is not solely a consequence of low dietary phosphorus intake. Instead, factors such as metabolic status, inflammation, and individual variation in mineral

metabolism may influence phosphate homeostasis (Bruins-van Sonsbeek & Corbee, 2024; Schook *et al.*, 2015).

Schook *et al.* (2015) proposed that hypophosphatemia in black rhinoceroses could also be driven by systemic metabolic disturbances, rather than simply dietary or environmental factors. In humans, inflammation and insulin resistance are known to impact phosphate homeostasis (Gaasbeek & Meinders, 2005), and similar mechanisms may be at play in rhinoceroses. Schook *et al.* (2015) hypothesized that insulin promotes cellular phosphate uptake, thereby lowering circulating extracellular phosphate levels. This reduction in serum phosphate may impair glucose uptake, worsening hyperglycaemia and stimulating further insulin secretion (Håglin *et al.*, 2001; Schook *et al.*, 2015). This feedback loop could contribute to a self-perpetuating cycle of metabolic imbalance involving hypophosphatemia, insulin resistance, and glucose dysregulation.

Their study also found that zoo-managed rhinoceroses had significantly higher levels of inflammatory markers and insulin compared to free-ranging individuals, despite similar glucose concentrations. These findings highlight the potential role of metabolic disturbances, especially inflammation and altered insulin dynamics, in disrupting phosphate regulation in captivity. Investigating these factors alongside dietary intake and UV exposure could yield valuable insights into optimal management strategies for maintaining rhinoceros health in zoological settings.

Conclusion

This study examined the correlation between serum 25(OH)D and plasma phosphate levels in black rhinoceroses housed at Rotterdam Zoo from 2020 to 2024, with a focus on the seasonal and yearly variations. A significant seasonal variation in serum 25(OH)D was observed, with levels peaking in summer and declining in winter, mirroring UV index fluctuations. Plasma phosphate concentrations displayed a less pronounced seasonal pattern, though winter values were significantly lower compared to other seasons. A statistically significant positive correlation was identified between log-transformed serum 25(OH)D and plasma phosphate, suggesting a potential role of vitamin D in phosphate homeostasis.

Despite this correlation, individual variation in both parameters underscores the complexity of their regulation, influenced by factors such as age, diet, and metabolic differences. Notably, phosphate levels stabilized in the later years of the study, potentially reflecting adjustments in dietary management. While plasma phosphate levels showed seasonal and individual variation, they remained within or near established reference ranges, and no clinical hypophosphatemia was observed.

These findings highlight the importance of refining dietary strategies and optimizing UV exposure to support the health and welfare of captive black rhinoceroses. However, the limited sample size and single-institution setting constrain the generalizability of these findings. Further research is warranted to investigate the mechanisms underlying vitamin D and phosphate metabolism in this species. Exploring additional regulatory factors, such

as calcium, PTH, and FGF-23, along with potential metabolic influences like inflammation and insulin resistance, could provide deeper insights into phosphate homeostasis.

Expanding such studies to multiple zoological institutions and increasing sample sizes could yield a more comprehensive understanding of phosphate regulation in black rhinoceroses. Ultimately, these findings contribute to evidence-based management strategies that enhance animal welfare and support conservation efforts for this critically endangered species.

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