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Physiological stress in eastern black rhinoceros (*Diceros bicornis michaeli*) as influenced by their density, climatological variables and sexes

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Abstract

It is important to understand the physiological stressors in animals especially for threatened species or intensively managed to improve their conservation and optimise their reproduction. We sought to understand changes in stress hormones (faecal glucocorticoid metabolites) in black rhinoceros (*Diceros bicornis michaeli*) in relation to population density and sex (intrinsic factors) and plant minerals, rainfall and land surface temperature (extrinsic factors). We used non-invasive faecal sampling techniques on animals of known sex, age and dominance in seven populations of contrasting population densities over 1 year. We measured variability in faecal corticosterone metabolites through radioimmunoassay and related them to population density, sex and faecal calcium, phosphorus, copper, zinc and potassium as characteristic of plant minerals, rainfall and temperature. We used linear mixed models (LMM) to analyse the data. We did not detect a significant relationship between physiological stress parameters and population density. However, we have indications that stress levels increased as rainfall and temperature increased and were correlated negatively and positively with concentration of faecal phosphorus and copper respectively; we found higher stress levels in females than in males. These results suggest that both intrinsic and extrinsic factors explain the variation in physiological stress observed in black rhinoceros.

KEYWORDS
black rhinoceros, corticosterone, density, faecal minerals, physiological stress, rainfall, sex, temperature

Résumé

Il est important de comprendre les facteurs de stress physiologiques chez les animaux, en particulier pour les espèces menacées ou gérées de manière intensive, afin d'améliorer leur conservation et d'optimiser leur reproduction. Nous avons cherché à comprendre les changements dans les hormones de stress (métabolites glucocorticoïdes fécaux) chez le rhinocéros noir (Diceros bicornis michaeli) en rapport avec la densité de population et le sexe (facteurs intrinsèques), et les minéraux végétaux,

les précipitations et la température de la surface du sol (facteurs extrinsèques). Nous avons utilisé des techniques d'échantillonnage fécal non invasives sur des animaux de sexe, d'âge et de dominance connus dans sept populations de densités contrastées sur une période d'un an. Nous avons mesuré la variabilité des métabolites fécaux de la corticostérone par dosage radio-immunologique et les avons associés à la densité de population, au sexe et au calcium, au phosphore, au cuivre, au zinc et au potassium fécaux, en tant que caractéristiques des minéraux végétaux, de la pluviométrie et de la température. Nous avons utilisé des modèles linéaires mixtes (LMM) pour analyser les données. Nous n'avons pas détecté de relation considérable entre les paramètres de stress physiologique et la densité de la population. Cependant, nous avons constaté que les niveaux de stress augmentaient avec les précipitations et la température et présentaient une corrélation négative et positive avec la concentration de phosphore fécal et de cuivre respectivement ; nous avons constaté des niveaux de stress plus élevés chez les femelles que chez les mâles. Ces résultats suggèrent que des facteurs intrinsèques et extrinsèques expliquent la variation du stress physiologique observée chez le rhinocéros noir.

1 | INTRODUCTION

Understanding physiological stress (defined here as the concentration of glucocorticoid secretion due to activation of the hypothalamic–pituitary–adrenal (HPA) system by external stimulus), is related to demography, environmental conditions and behaviour and is important in population performance studies (Creel, 2001; Wingfield & Sapolsky, 2003). Enclosed or intensively managed wildlife populations may be prone to higher physiological stress levels compared to free-ranging populations due to higher densities. The relationship between physiological stress and population density (Selye, 1946) was first advanced by Christian (1950) who hypothesised activation of the HPA in meadow voles (*Microtus* spp.) due to high density. Li et al. (2007) also reported high levels of adrenocortical secretion in Pére David's deer (*Elaphurus davidianus*) living in small enclosures at high densities. Chronic physiological stress levels lead to low survival probability, immunosuppression, diseases (Christian, 1950), loss of body condition and poor reproductive capability (Kirby et al., 2009; Singer & Zumoff, 1992; Wasser & Starling, 1988) or disruption of metabolism and gastrointestinal functions especially where quantity and quality of food resources are lowered by high population densities. High densities of megaherbivores putatively lead to physiological stress in individuals at 70%–80% of the population maximum stocking density (e.g. McCullough, 1999; Owen-Smith, 1988). In captivity, social stressors may cause chronic stress in black (*Diceros bicornis*) and white rhinos (*Ceratotherium simum*) contributing to their captive-population sustainability problems (Carlstead & Brown, 2005). It is, however, not always that low cortisol is an indicator of low stress levels. Linklater et al. (2010), for example, found black rhinoceros and male white rhinoceros corticoids declined below pre-translocation (stressor) values. Their study associated the low corticoids with suppressed levels of androgens and progestins with increased time in captivity.

Food quality may affect stress hormones too. For example, dietary fibre and plant minerals (Breman & De-Wit, 1983; Lester et al., 2010)

as influenced by plant phenology (Huber et al., 2003; Millspaugh et al., 2001; Millspaugh & Washburn, 2004) can affect gut microbiota which has been implicated in a variety of stress related conditions in animals (Wasser et al., 1993). Food factors as influenced by population density (Coomes et al., 2003) could override other stressors.

There are sex differences that might take any direction, and be different at different times, for example depending on reproductive cycles, lactation or breeding seasons among others ((e.g. Carlstead & Brown, 2005; Kudielka & Kirschbaum, 2005; Millspaugh et al., 2001; Munshi-South et al., 2008). The sexual differences in stress levels in humans are attributed to male and female brain dimorphisms (Luque et al., 1992; Pinos et al., 2001) and to ovarian cycles (Ter Horst et al., 2009). Social species are more vulnerable to physiological stress than territorial species (Creel et al., 2013; Engh et al., 2006; Kuznetsov et al., 2004; Weingrill et al., 2004). Less dominant animals are generally more physiologically stressed than the dominant ones (Creel, 2001) with some exceptions such as the male North American elk (*Cervus elaphus*) attending to a group of females tending to have higher levels of stress than subordinates (Chen et al., 2009; Millspaugh et al., 2001).

Seasonal changes, rainfall and temperature can modulate basal and elevated glucocorticoid secretion in birds, mammals, amphibians and reptiles in a predictable pattern during the annual cycle (Harper & Austad, 2001; Millspaugh et al., 2001, 2002; Millspaugh & Washburn, 2004). For example, faecal glucocorticoid concentrations among free-ranging African savannah elephants (*Loxodonta africana*) was found to be higher in the dry season than in the wet season, a possible physiological stress resulting from a decline in food quantity and quality (Viljoen et al., 2008; Wato et al., 2016, 2018), whereas in North American elk faecal glucocorticoid have been reported to be lower in winter and higher in summer which could be triggered by high temperatures and human disturbance (Millspaugh et al., 2001) or by food availability that trigger breeding activity that is associated with high glucocorticoid. Similar observation of high faecal

glucocorticoid has been made in other non-seasonal but resource-triggered breeding in rhinos.

Here, we report physiological stress responses of a mega-herbivore, black rhinoceros (*Diceros bicornis michaeli*; Prins, 1990), in contrasting population densities, rainfall, temperature, plant mineral concentrations as measured via faecal minerals and influence of sexes. We use faecal sampling because data on metabolic products consisting of digestive tract cells, secretions and microbes can be gleaned from the faeces non-invasively without sampling impacting stress measures (Millspaugh & Washburn, 2004; Möstl & Palme, 2002). Excreted hormones via faeces also represent average values pooled over time, rather than a single point-in time measure. More so, faecal and dietary mineral concentrations have been shown to be directly correlated for black rhinoceros in captivity (Clauss et al., 2007) and in the wild (Okita-Ouma, Pettifor, et al., 2021). This offers an opportunity to understand the influence of diet quality on corticosterone metabolite levels as measured in faecal material. Finally, there is uncertainty in the direction of the impact caused by these stressors. The result depends on several factors thus worth understanding for this critically endangered species.

We chose the Critically Endangered black rhinoceros (Emslie, 2020) for this study for the following reasons: it is a selective browser that significantly impacts its habitat especially if enclosed in high density (e.g. Luske et al., 2009) which may lead to an elevated physiological stress either directly because of elevated social interactions in an otherwise rather solitary species or indirectly through reduction in resource quality. Secondly, its population regulation is bottom-up through food resources as opposed to top-down because adult black rhinoceroses are nearly invulnerable to African predators such as spotted hyaena (*Crocuta crocuta*), lion (*Panthera leo*) or leopard (*P. pardus*; see review in Emslie & Adcock, 2013). This makes it possible to detect density dependence effects by examining its food quality and quantity. Thirdly, its reproduction is largely resource driven and even though the nulliparous females may appear to synchronise their oestrus compared to compared to their multiparous counterparts (Garnier et al., 2002), it may therefore not have significant seasonal variation in stress hormone secretion during pregnancies.

We hypothesise that: (i) Physiological stress levels will increase as population density increases. (ii) A negative correlation between corticosterone metabolite and tail end of rainy season when food resources are abundant but a positive correlation with average land surface temperature as hot seasons are generally dry seasons with scarcity of food and water. (iii) A negative correlation between corticosterone metabolite and concentration of dietary minerals as measured through faecal calcium (Ca), phosphorus (P), copper (Cu), Zinc (Zn) and potassium (K). (iv) Higher physiological stress levels in female than in male black rhinoceros as observed in many mammalian species.

2 | METHODS

We conducted the study in Aberdare National Park ('Aberdare'), Maasai Mara National Reserve ('Mara'), Ngulia Rhino Sanctuary ('Ngulia'), Ol Jogi Pyramid ('Ol Jogi'), Ol Pejeta Conservancy ('Ol Pejeta'), Tsavo East National Park ('Tsavo East') and Tsavo West National Park IPZ ('Tsavo West IPZ'; Figure 1). The sites were of contrasting black rhinoceros population densities (Table 1) and of varying ecological characteristics, rainfall, soils and vegetation as described in Okita-Ouma, Pettifor, et al. (2021). Generally, in the horn of Africa where the study sites are located, land surface temperature is inversely correlated with precipitation (Abera et al., 2019; KMD, 2020). We collected 198 faecal samples from 29 males and 189 faecal samples from the 31 females for mineral analysis as proxies of dietary quality. Of these, we assayed 151 faecal samples from 25 males and 137 faecal samples from 20 females for corticosterone metabolites resulting in a mean of 6.5 faecal samples per animal for mineral nutrients and a mean of 6.4 faecal samples per animal for corticosterone metabolites, both spread over the 13 months of data collection. Ngulia, Ol Jogi and Ol Pejeta exceeded their estimated maximum stocking densities, whereas Aberdare, Maasai Mara, Tsavo East and Tsavo West IPZ had densities below the estimated maximum stocking density as given by Adcock et al. (2007) (Table 1).

2.1 | Field sampling and collection procedures

Specialised rhinoceros monitoring personnel collected demographic data which underwent quality control processes prior to storing for analyses in the central Kenya Black Rhino Information System (Amin et al., 2001). The personnel's day-to-day work is to monitor individual animals with unique code numbers, names or markings such as through ear-notch patterns. We deployed a non-invasive faecal sampling technique to collect fresh (<18 h old) samples of known adult (>7 years old) black rhinoceroses for 13 months from July 2010 to July 2011. Sample collection was between 0600 h and 1200 h East African Time. We removed visible contaminants like surface soil particles or flies but avoided samples contaminated with urine or rainwater. We mixed thoroughly by hand in gloves to achieve an even distribution of metabolites within the faeces sample (Millspaugh & Washburn, 2003) put two samples (one for backup purposes) of about 30 g into airtight sampling bottles. We transported samples in ice-packed cooler box before transferring them into a −20°C freezer for storage. We minimised contamination of samples by not re-using handling equipment such as gloves, polythene sheets and sampling bottles.

2.2 | Sample preparations and analyses

We sent subsamples in two 'lots'/batches to University of Missouri USA for hormonal analyses while faecal mineral concentration were analysed at the industrial laboratories of Ministry of Mines and Geology in Nairobi. The first 'lot' contained samples collected between July 2010 and March 2011, a period of lower rainfall than between March 2011 and July 2011 when >95% of samples in the second 'lot' were collected. The subsamples were
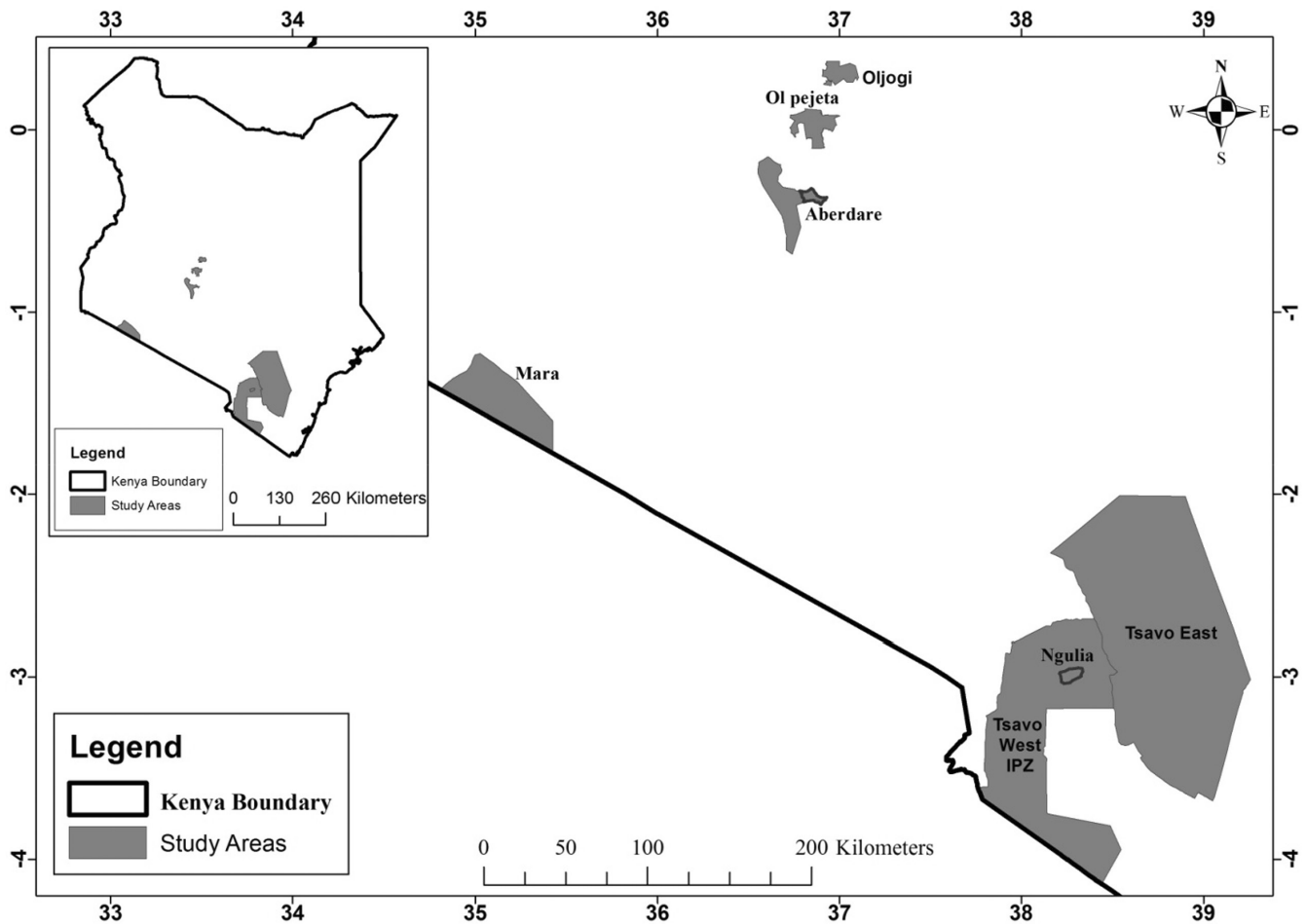
FIGURE 1   Locations of the seven black rhinoceros study sites in Kenya. Tanzania to the south, and the Indian ocean to the south east.

tested for Foot-and-Mouth Disease (FMD) in Nairobi and every approximately 5 g of faecal samples were soaked in 3 mL of 2% acetic acid for health and safety reasons before they were shipped to USA. Treatment of faecal samples with 2% acetic acid meets import requirements by the U.S. Department of Agriculture and has a minimal impact on hormone values (Millspaugh et al., 2003). The subsamples shipped to the USA were freeze-dried in a BOC EDWARDS, Minifast 04 freeze drier for at least 48 h to zero moisture at the Kenya Medical Research (KEMRI) Laboratories in Nairobi. Faecal samples were rubber-cocked in freeze-drying bottles and sealed with aluminium caps to prevent absorption of moisture and shipped to USA on dry ice. 'lot'. We duplicated sets of frozen subsamples to the Ministry of Geology and Mines Laboratory in Nairobi for faecal mineral analyses.

2.3 | Stress hormonal assay

We placed approximately 0.2 g of dried faeces in a test tube with 2.0 mL of 90% methanol and vortexed in a multi-tube vortex mixer for 30 min at high speed then centrifuged at ~1900g for 20 min and saved the supernatant for storage at −20°C until analysed using radioimmunoassay procedures. We measured corticosterone

metabolites in black rhinoceros faeces using a commercially available corticosterone I^{125} double-antibody radioimmunoassay kit (Cat. #07120103, MP Biomedicals). The manufacturer's method for the corticosterone I^{125} radioimmunoassay was followed, except that the volumes of all reagents were halved (Wasser et al., 2000).

We ran a standard assay validation including assessment of parallelism, recovery of exogenous analyte, intra- and inter-assay precision and assay sensitivity (Grotjan & Keel, 1996; Jeffcoate, 1981; O'Fegan, 2000) to confirm the assay accuracy and precision of the measured corticosterone metabolites in the rhinoceros faeces. We conducted parallelism and recovery of exogenous corticosterone validation assays on two pooled faecal extract samples (each pool consisted of faeces from three individuals). Parallelism ensures the assay maintains linearity under dilution, and recovery of exogenous corticosterone verifies accurate measurement throughout the working range of the assay (Jeffcoate, 1981). We added exogenous corticosterone to the low and high-pooled faecal extracts to obtain corticosterone values under higher dilution levels. We used tests for equal slopes (parallelism) to determine if ^{10}log transformed curves of serially diluted pooled faecal extracts were parallel to ^{10}log transformed corticosterone standard curves. We calculated intra-assay variation by averaging the coefficient of variation (CVs) of replicate tubes from 20 randomly chosen samples (Jones & Payne, 1997).

TABLE 1 A summary of attributes measured within study areas.

Study area	Area (km²)	N	Estimated maximum stocking density (D_{max}) (Adcock et al., 2007)	Density (N.km⁻²)	Relative density to D_{max} (%)	N animals for corticosterone metabolite		N animals for faecal mineral concentration	
						Males	Females	Males	Females
Ngulia	92	69	0.41	0.75	185.2	5	0	5	6
Ol Jogi	50	21	0.23	0.42	183.4	5	5	5	5
Ol Pejeta	300	87	0.31	0.29	94.5	5	6	5	6
Mara	1510	37	0.07	0.02	2.9	2	3	2	4
Aberdare	70	8	1.00	0.11	11.0	2	3	2	3
Tsavo East	1800[a]	31	0.41	0.02	4.9	4	3	4	5
Tsavo West (IPZ)	1200[a]	10	0.41	0.01	2.5	2	0	3	2

Note: Sizes of study areas, black rhinoceros population (N), population densities (N.km⁻²), an estimated maximum stocking density (D_{max}), relative densities in comparison to D_{max}, and the number of animals repeatedly sampled for faecal corticosterone metabolites and mineral measurements.

[a]The area used by black rhinoceros based on field reports by rangers within Tsavo East NP of approximately 11,000 km² and Tsavo West NP of approximately 9000 km².

The assay accurately and precisely measured faecal glucocorticoid metabolites in black rhinoceroses' samples. Serial dilutions (1:4 up to 1:128) of rhinoceros faecal extracts yielded displacement curves that were parallel (all $p > 0.5$) to the corticosterone standard curve (Figure 2) Mean recovery of added exogenous corticosterone (range = 0.25–1.25 ng/mL) was 110.9% at 99% CL for $n = 10$). Acceptable recovery of exogenous corticosterone (within 90%–110%) and demonstration of parallelism suggested no sample matrix effects (Grotjan & Keel, 1996; Jeffcoate, 1981; O'Fegan, 2000). Assay sensitivity was 1.25 ng/g. The manufacturer reported cross-reactivity of the antisera as 100% with corticosterone and <1% for other steroids. Inter-assay variation for the assays was 6.2% and average intra-assay variation was 1.3%.

2.4 | Mineral analyses

We followed standard procedures of Plank (1992) for preparation of samples for determination of mineral concentration in plants. Detailed description on the preparation of samples and analyses of faecal Cu, Zn, P, Ca and K are in Okita-Ouma, Pettifor, et al. (2021).

The precision at 95% confidence level of measures as obtained from the standard parallelism curves were: ±0.002 ppm for Cu; ±0.005 ppm for Zn; ±0.05 ppm for P; ±0.05 ppm for Ca and ±0.02 ppm for K. All measured minerals from black rhinoceros faeces yielded results parallel to the standard curves (F test, all $p > 0.05$).

2.5 | Statistical procedures

We used rainfall and temperature to characterise seasonality (LSASAF, 2010; Tchuenté et al., 2011) and derived estimates from raster images of 8 km resolution for rainfall and 4 km resolution for temperatures for 2010 to 2011 inclusive (http://www.cpc.ncep.noaa.gov/products/fews/AFR_CLIM/afr_clim.shtml). We used Hawth's Tools for ArcMap9.3.1© and ArcGIS™ software to extract specific rainfall and temperature estimates from satellite raster images by overlaying the boundary of each study area on the raster
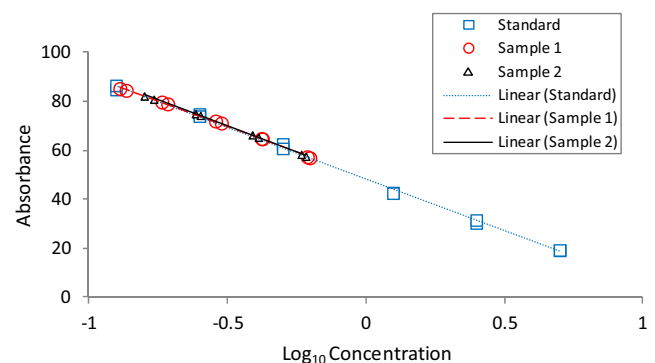

FIGURE 2 Parallelism curve used to assess accuracy and precision of corticosterone metabolites measured in black rhinoceroses' faeces for this study.

images. Even though the satellite-derived measurements of rainfall and Land Surface Temperatures (LST) are similar to field measurements (LSASAF, 2009), we reconfirmed this by correlating satellite-derived rainfall and the reliable rain-gauge rainfall for two study sites since it was critical for our analysis (Appendix S1). We did not expect rainfall and temperature to have an immediate influence on food resources and consequently physiological stress levels and therefore we lagged their effects by counting 29 days back for a 1-month lag and 59 days preceding faeces deposition for a 2-month lag.

We imputed missing mineral values for 11 faecal samples by fitting two-way ANOVA models per mineral, using area, month and area*month as factors, and replacing the missing values by predictions from the two-way ANOVA models.

We used linear mixed models (LMM) to analyse the data by applying the procedure MIXED (Littell et al., 2006) of the SAS® software Version 9.4. We used mixed models due to the complex data collection process, resulting in repeated measurements on areas, black rhinoceroses (per area), faecal samples (per rhinoceros) and measurements of corticosterone metabolites in two 'lots' at different times whereby samples in the first 'lot' were assayed with two replicates per sample whereas samples in the second 'lot' were assayed once. 'Lot' had the potential to be a confounder of effects of other factors (seasonal effects, minerals, corticosterone metabolites) because the two 'lots' largely coincided with dry and wet seasons respectively. For that reason, we fitted models without and with 'lot' and compared estimates and significances.

Corticosterone metabolites values were ^{10}log transformed. The fixed part of the mixed model included effects for explanatory variables related to area (black rhinoceroses' density), area-time combination (rainfall and temperature), rhinoceros (sex) and rhinoceros-time combination (minerals measured in the faeces) in the mixed model for log (corticosterone). The random part of the mixed model included effects for different sources of variation: area, rhinoceros, dung sample and residual error. The residual error variance quantifies the variability of the duplicates of dung samples analysed in the first 'lot' of samples.

We took the following steps to build a parsimonious model to explain variability in observed corticosterone metabolites:

Step 1: As starting model we fitted a mixed model with fixed variables: density, sex, the set of five mineral nutrients (Ca, P, Cu, Zn, K all ^{10}log transformed) and the three unlagged climatological variables (maximum and minimum LST, rainfall), and random effects for area, rhinoceros, faecal sample and random effect for error.

Step 2: Determination of best lag for climatological variables. As weather effects from the past may affect the present, the best lag for temperature and rainfall was determined, by evaluating mixed models comparing −2 log likelihood (−2LL) containing combinations of unlagged, 1-month lagged and 2-month lagged climatological variables. From a plant ecophysiological perspective, we considered likely that cumulative rainfall and temperature effects may influence the nutrient content of plants (e.g. Ahrestani et al., 2011; Okita-Ouma, van Langevelde, et al., 2021); hence, we lagged both rainfall and temperature. The model with lowest -2LL was chosen. Climatological

variables needed to be statistically significant ($p < 0.01$) to remain in the model.

Step 3: We used backward elimination to select the important minerals, starting with all five minerals in the model and removing the insignificant ones. We also applied forward selection, starting with the model without minerals and adding individual minerals one by one, to check whether the same subset was selected as found for the backward elimination. The p-value to retain a variable in the model was set at 0.01.

Step 4: Since variability of faecal corticosterone may be different for males and females, we compared models with constant and sex dependent variance components with likelihood ratio tests.

Step 5: To judge the potentially confounding effect of 'lot', we fitted the resulting model with and without random effects of 'lot'. We report the results from both models, comparing the coefficients of the explanatory variables in the models without and with 'lot'. During the model construction phase (steps 1–4) we fitted all LMM's by maximising the full likelihood, using the resulting -2LL as criterion for model comparison, for example for hypothesis testing. The final models (step 5) were fitted using restricted maximum likelihood, applying approximate F-tests for fixed with the method by Kenward & Roger (1997).

3 | RESULTS

The final linear mixed model obtained for corticosterone (symbolised by 'y'; without 'lot' as explanatory factor) was:

$$\log\left(\underline{y}\right) = \beta_0 + \beta_1 \text{ density} + \underline{\text{area}} + \beta_2 \text{ sex} + \underline{\text{rhinoceros}}$$
$$+ \beta_3 \text{ 2 month rainfall lag} + \beta_4 \text{ 2 month max temperature lag}$$
$$+ \beta_5 \log(\text{phosphorous}) + \beta_6 \log(\text{copper}) + \underline{\text{faecal sample}} + \underline{\text{error.}}$$

With (y) corticosterone, all underlined terms represented random effects with variance components: var($\underline{\text{area}}$) = σ^2_a, var($\underline{\text{rhinoceros}}$) = σ^2_r, var($\underline{\text{faecal sample}}$) = σ^2_d and var($\underline{\text{error}}$) = σ^2_{eM} for males and σ^2_{eF} for females.

The results are summarised in Table 2 based on the final model. In Table 3 the same model is fitted but with an extra random effect for 'lot'.

The conclusions from the two models, without (Table 2) and with 'lot' (Table 2) are the same for density and sex as explanatory variables. Density is not significantly related to cortisol ($p > 0.05$) whereas females show higher cortisol levels than males ($p < 0.05$). However, for the remaining explanatory variables p-values in the model with 'lot' are considerably larger than in the model without 'lot'. For lagged max temperature and rainfall the estimated coefficients are approximately 2.5 times smaller in the model with 'lot', while the standard errors are roughly the same, leading to smaller t-values and larger (non-significant) p-values in the model with 'lot'. For minerals log phosphorus and log copper the estimated coefficient are 5 to 6 times smaller in the model with 'lot', while standard errors show only small changes, again leading to smaller t-values and

much larger, non-significant *p*-values. Notice that the signs of the effects are consistent for the models without and with 'lot' as explanatory variable: positive trends remain positive, negative trends remain negative in the model with 'lot'.

We conclude that the relationships between cortisol and climatological variables and minerals phosphorus and copper, shown in Table 2, may partly be explained by variable 'lot', as shown in Table 2. This may be due to confounding of 'lot' with those variables, although variance inflation factors of all regressors are small (largest value is 1.83).

Even though corticosterone metabolite levels increased with increases in density, this was not significant [($b_1 \pm se(b_1)$): $5.43 \times 10^{-2} \pm 7.93 \times 10^{-2}$]. Rainfall effects on corticosterone metabolite levels were not apparent at the onset of rainfall but a potential lag effect of rainfall was detected. In the model without 'lot' we found the 2-month lag of rainfall to have a strong significant positive relationship with corticosterone metabolite [($b_3 \pm se(b_3)$): $2.52 \times 10^{-3} \pm 7.0 \times 10^{-4}$]. Corticosterone metabolite levels peaked in May, 2 months into the rainy season (Figure 3a).

As expected, corticosterone metabolite levels increased with increase in temperature. However, it was the 2-month lag of maximum land surface temperature in the model without 'lot' that had a significant positive relationship with corticosterone metabolite [($b_4 \pm se(b_4)$): $1.22 \times 10^{-4} \pm 2.2 \times 10^{-5}$] (Figure 3b). This model also showed that ^{10}log P and ^{10}log Cu concentrations had strong

statistical explanatory power after all minerals except for P and Cu were removed from the model after backward elimination and forward inclusion of important minerals. Concentration of P in faeces was found to have a negative and significant relationship with corticosterone metabolite [($b_5 \pm se(b_5)$): $-1.26 \times 10^{-1} \pm 3.77 \times 10^{-2}$] while concentration that of Cu had a significant positive relationship with corticosterone [($b_6 \pm se(b_6)$): $2.09 \times 10^{-1} \pm 5.42 \times 10^{-2}$]. In the model with lot these climatological and mineral variables did not show significance anymore; however, the signs of the effects remained the same. Sex influenced corticosterone metabolite levels in two ways. First, females had significantly higher corticosterone metabolites than males [($b_2 \pm se(b_2)$): $5.22 \times 10^{-2} \pm 2.21 \times 10^{-2}$] and secondly, the variability in measurements of duplicates within female faecal corticosterone metabolite was significantly higher than in male faecal corticosterone metabolite: (females $S_{eM}^2 = 7.6 \times 10^{-3}$, males $S_{eM}^2 = 3.8 \times 10^{-3}$, $p = 0.01$).

We did not find 1-month lag in rainfall or temperature a strong statistical explanatory variable and therefore even though it was tested, its results are not reported.

4 | DISCUSSION

The 'lots' coincided with seasonality, where 'lot' 1 were generally samples collected in dry season whereas 'lot' 2 were samples

TABLE 2 Results of the linear mixed model obtained for variability in corticosterone metabolites in faeces of black rhinoceros as explained by population density, climatological variables and faecal mineral nutrients characterising plant nutrients in 2010/2011 in the study sites.

Effect		Estimate	Standard error	df	t value	Pr > |t|
Intercept		1.01	2.17×10^{-1}	143	4.64	<0.0001
Density (rhinoceroses/km^2)		5.43×10^{-2}	7.93×10^{-2}	4.57	0.68	0.53
Max. Temp (°C; 2-month lag)		1.22×10^{-4}	2.2×10^{-5}	224	5.52	<0.0001
Rainfall (mm; 2-month lag)		2.52×10^{-3}	7.0×10^{-4}	291	3.61	0.0004
Sex (Female vs. Male)	Female	5.52×10^{-2}	2.26×10^{-2}	39.1	2.44	0.019
Log Phosphorus (mg/kg)		-1.26×10^{-1}	3.77×10^{-2}	305	−3.33	0.001
Log Copper (mg/kg)		2.09×10^{-1}	5.42×10^{-2}	295	3.85	0.0001

Note: Two months lagged maximum temperature and rainfall, and phosphorus and copper significantly explained the variability in faecal corticosterone metabolites ($p < 0.000$). Population density did not significantly explain the observed variability in faecal corticosterone metabolites ($p = 0.53$). Females had significantly higher corticosterone metabolites than males ($p = 0.019$), (−2 log likelihood (−2LL) = −510.8).

TABLE 3 Results of the linear mixed model with extra random effect for 'lot' (−2LL = 541.4).

| Effect		Estimate	Standard error	df	t value	Pr > |t|
Intercept		1.11	2.22×10^{-1}	27.5	5.01	<0.0001
Density (rhinoceroses/km^2)		5.24×10^{-2}	4.06×10^{-2}	3.06	1.29	0.29
Max. Temp (°C; 2-month lag)		4.7×10^{-5}	2.5×10^{-5}	27	1.91	0.067
Rainfall (mm; 2-month lag)		1.08×10^{-3}	7.25×10^{-4}	228	1.49	0.1363
Sex (Female vs. Male)	Female	5.22×10^{-2}	2.21×10^{-2}	37	2.37	0.023
Log Phosphorus (mg/kg)		-2.69×10^{-2}	4.08×10^{-2}	305	−0.66	0.51
Log Copper (mg/kg)		3.17×10^{-2}	5.90×10^{-2}	293	0.29	0.59
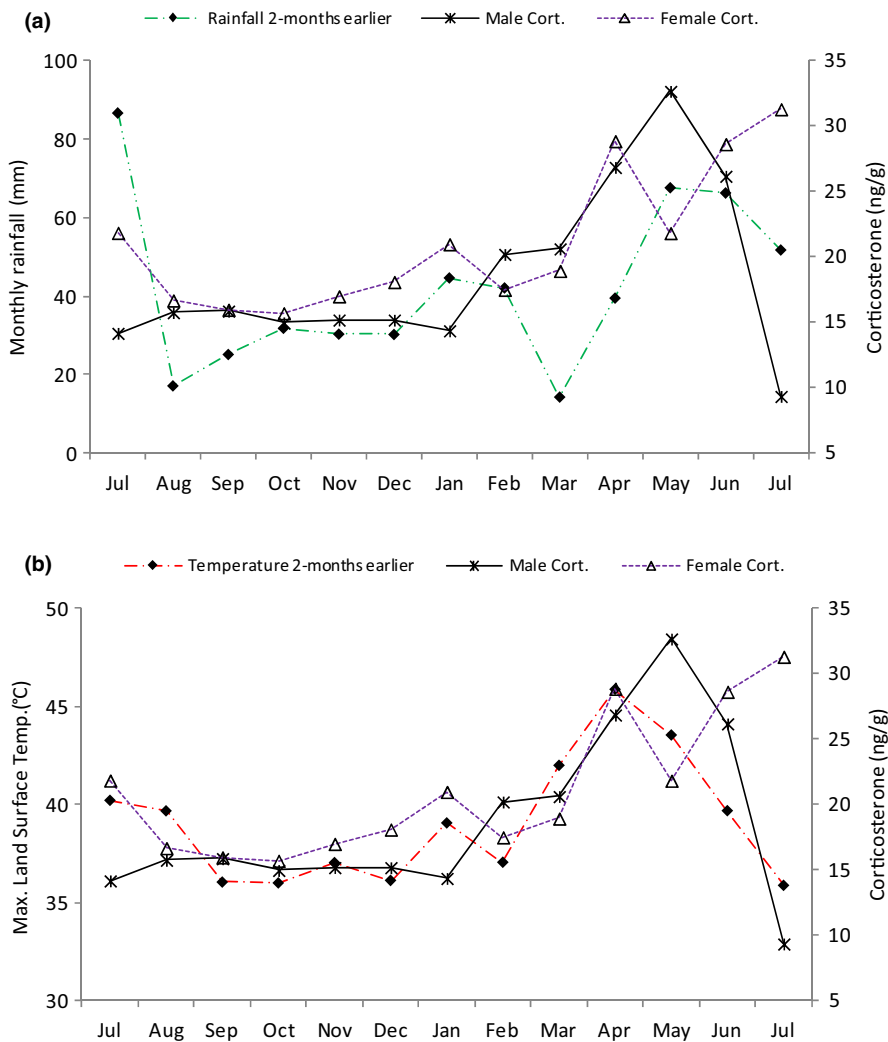
FIGURE 3 (a) Relationships between 2-month lag of rainfall and corticosterone metabolite levels in male and female black rhinoceros, 2010/2011 in seven study sites in Kenya. Corticosterone peaked 2 months following a peak in rainfall. (b) Relationships between 2-month lag maximum land surface temperature and corticosterone metabolite levels in male and female black rhinoceros in 2010/2011 in seven study sites in Kenya. Corticosterone peaked 2 months after a peak in land surface temperature.

collected during rainy wet season. Dry and wet seasons in the study areas are generally associated with high and low temperatures respectively. Rainfall is also known to correlate with plant mineral concentrations. The observed differences in the results of the two models without and with 'lot' on weather and mineral effects as opposed to density and sex can be attributed to the correlations of the 'lots' with climatical variables. We therefore discuss our results with this correlation in consideration.

4.1 | Population density effects on corticosterone levels

To some, mammalian megaherbivores are thought to be controlled by density feedback mechanisms particularly when population near or attain maximum stocking levels (e.g. Eberhardt, 2002; Law et al., 2013; McCullough, 1999; Owen-Smith, 1988, 2011; Pastor et al., 2006). Other scholars argue that mammalian mega-herbivore populations in situ subject to sufficient environmental variability are governed by fundamentally different processes other than density (e.g. Fowler, 1981; Gough & Kerley, 2006; Morgan et al., 2009). Our results in both models without and with 'lot' did not show significant

effects of black rhino density on corticosterone metabolite levels despite high densities of Ngulia, Ol Jogi and Ol Pejeta relative to estimated maximum stocking levels (Adcock et al., 2007) exceeding the 70–80% of maximum stocking density supposedly resulting in negative density feedback (Caughley, 1976; Kirkwood et al., 1994; McCullough, 1992; Robinson, 2000). Our findings were thus contrary to Christian's (1950) hypothesis that high population density leads to physiological mechanisms that trigger the HPA system to secrete elevated corticosterone metabolites. At the same time, our results also do not suggest that animals living at high population densities have particularly low corticosterone metabolites, which has been suggested as an indication for 'distress' in black rhinos (Linklater et al., 2010). Apparently, the animals appear to be unaffected, in terms of their adrenergic stress response, by these levels of population density differences. Kuznetsov et al. (2004) also found similar results in midday gerbils (*Meriones meridianus*), whereas Evsikov et al. (1999) found an inverse relationship between population density and physiological stress levels in water vole (*Arvicola terrestris*). Territorial species are generally less prone to physiological stress compared to social species at high population densities (Creel et al., 2013; Kuznetsov et al., 2004) as reported in the territorial gerbil (*Meriones unguiculatus*) when grouped in mixed-sexes compared

to when grouped in same-sex (Hull et al., 1973). In these respects, the territorial nature of the black rhinoceros, the fact that males allow females into their territories, and the occasional social tolerance at shared resources for example at drinking-water points, may partly explain why there was no significant effect of population density on stress levels. Large browsers also have sufficient behavioural plasticity to adjust their diet to cover nutritional requirements when their density increases.

4.2 | Climate variable effects on corticosterone levels

4.2.1 | Rainfall

The strong and direct relationships between physiological stress levels in black rhinoceros and the 2-month lag of maximum temperatures (see below) and the 2-month lag of rainfall in the model without 'lot'. Rainfall is an important factor that seasonally modifies a range of influential abiotic and biotic factors in the environment. It leads to forage availability and mobilises exchangeable plant available nutrients in the soil for uptake by plants, which in turn affects physiological processes within an animal.

The direct relationship between corticosterone and the 2-month lagged rainfall can be attributed to the expected decreased consumption of fibre by browsers during rainy/growing season because of abundance of forbs with high amounts of proteins and carbohydrates and little fibre (Ndeereh et al., 2012), yet faecal corticosterone metabolite concentration should be diluted and lowered by high fibre intake. This result is elevated corticosterone levels soon after the rains. More so, black rhinoceros breeding activity is resource-triggered, a normal rainfall after a normal dry season leads to a state of anabolism where also the adrenergic system is activated leading to more reproductive activity as frequently described in non-seasonal breeders in better body condition (Radeke-Auer et al., 2022).

4.2.2 | Land surface temperatures

Our findings showed that levels of corticosterone metabolites in black rhinoceros increased with increases in maximum land surface temperatures (LST) with a 2-month lag in the model without 'lot'. This may be explained by the temperature-rainfall corelations and their effects on availability and quality of food resources are not instantaneous but lagged. And as already discussed in the preceding section, forage may increase secretion of corticosterone in different ways. LST increases soil temperature and availability of total nitrogen, ammonium and phosphates to plants (Kelly, 1993) at the onset of rainfall. Quality (Barbosa et al., 2014; Singh & Singh, 2004) and quantity (Olff et al., 2002; Owen-Smith, 1994, 2008; Sankaran et al., 2008) of browsing forage (twigs and leaves) tend to increase with increase in water availability. In the model with 'lot' just as was in the case of rainfall where temperatures

had no significant effects on corticosterone, 'lot' as a confounding effect can be explained by its correlation with the climatic variables. Moreso, increases in temperature has been reported to stimulate the pituitary gland to gonadotrophic activity (Wells & Zalesky, 1940) to increase levels of secreted corticosterone metabolites in free-ranging North American elk (*Cervus elaphus*) (Millspaugh et al., 2001).

4.3 | Mineral effects on corticosterone levels

In our study, we assumed that concentration of minerals in faecal samples was a proxy for the concentration of dietary minerals as shown by Clauss et al. (2007), Okita-Ouma, Pettifor, et al. (2021) and as known to correlate with rainfall. P and Cu, as proxies of quality of diet, showed significant correlations with corticosterone metabolite levels in the model without 'lot'. As expected, P was negatively correlated with the stress levels. This could be linked to the reported inverse correlation between precipitation and P availability in soil (Hou et al., 2018). As for the positive correlation between Cu and stress levels, we can only speculate. Okita-Ouma, Pettifor, et al. (2021) found a positive relationship between dietary Cu and faecal copper but did not find any evidence that Cu had a negative effect on the density of black rhinos in Kenya. Even though exposure to Cu may lead to toxicity in animals and may induce responses through release of stress hormones, we have no indication that Cu levels in the diets of the rhinos reached a level that could be flagged as 'risky'. Hence, the corelation between faecal Cu levels and stress indicators may be spurious or the causality may even be the inverse (i.e. high levels of stress hormone inducing more copper excretion). We are strongly aware that observation precedes theory in ecology and physiology like in many sciences (e.g. Gordon & Prins, 2019; Prins & Gordon, 2014; Zinman, 1978). Our results may contradict generalised statements about the wet season being the best for tropical herbivores, thus invalidating applying such rules to black rhinoceros and perhaps other Perissodactyla and even Afrotheria.

4.4 | Effects of sex

As hypothesised, females had higher corticosterone levels than males. This even though was reported for the first time for black rhinoceros in situ has been found to be common in many other mammalian species (e.g. Kudielka & Kirschbaum, 2005). Female African forest elephants in the Gamba Complex of Gabon reportedly had higher glucocorticoid levels than their male counterparts when exposed to disturbance (Munshi-South et al., 2008) even though male Asian elephants (*Elephas maximus*) were observed to be more stressed than females in human dominated landscapes (Vijayakrishnan et al., 2018). Despite our study not measuring oestrogen levels in females, they had a calf at foot, pregnant, on oestrus or a combination of the three status corroborated from their optimal breeding performance (Okita-Ouma, van Langevelde,

et al., 2021) and information pertaining to births, deaths and calving periods in the Kenya Black Rhino Information System (Amin et al., 2001) thus physiologically elevating stress hormones (e.g. Lyimo et al., 2000; Radeke-Auer et al., 2022). We note here that a variety of stressful behaviours are associated with reproduction in male mammals too (see review in Reeder & Kramer, 2005). Additionally, stress levels can be linked to behavioural differences. Less dominant animals and less territorial animals have been shown to generally exhibit higher physiological stress levels than the dominant animals (Creel, 2001) or the territorial animals (Kuznetsov et al., 2004) except for some exceptions (e.g. Millspaugh et al., 2001). In our case, we posit that the female black rhinoceros is behaviourally less dominant and less territorial compared to the males (see review in Emslie & Adcock, 2013) and therefore exhibited higher levels of physiological stress.

5 | CONCLUSION

We show that levels of faecal corticosterone metabolites are not density dependent in the black rhinoceros' populations in this study. Density effect is probably overridden by the influence of climatological variables where increases in 2-month lagged rainfall and Least Surface Temperature are correlated with increases in the levels of physiological stress. These two climatic variables influence quantity and quality of food resources that in turn influence physiological stress as shown in the correlation between corticosterone metabolites and concentrations of phosphorus and copper as proxies for dietary minerals. We demonstrate that corticosterone (stress) levels are higher in female than in male black rhinoceros even though the exact causal stressor for sexual difference was not identified in our study. Our results provide a range of both basal and elevated corticosterone metabolites in black rhinoceros for future studies and demonstrate that stressors of black rhinoceros in situ in the study areas are both intrinsic and extrinsic.

CONFLICT OF INTEREST STATEMENT
The authors declare no conflict of interest in relation to this study.

DATA AVAILABILITY STATEMENT
All data used in this research is available on a request to the lead author.

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