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Monthly plasma oxytocin samples detect lactational but not social management or behavioral variation in *ex situ* black and white rhinoceroses

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ABSTRACT

The neuropeptide hormone oxytocin is essential for uterine contractions and lactation, facilitates reproductive and social behaviors, and may also regulate stress and psychological state. Plasma samples were collected monthly from black (*Diceros bicornis*; n = 19 males, 17 females) and white (*Ceratotherium simum*; n = 20 males, 40 females) rhinoceroses housed across 33 North American facilities. Focal animal behavioral observations were conducted twice, ~6 months apart, for three to five days each time. Plasma oxytocin was extracted via proteomic reduction, alkylation, and precipitation methods. Successful analytical validation of the enzyme immunoassay consisted of a parallel displacement curve between serially diluted standards and pooled extracts, linearity across dilutions, and 96% recovery of spiked synthetic hormone. Monthly peripheral plasma oxytocin concentrations did not differ between black and white rhinoceroses but were positively associated with age in the former ($p = 0.049$). In lactating white rhinoceroses, oxytocin increased from 0 to 365 days postpartum ($p < 0.001$), serving as a biological validation of the assay. There were no significant associations detected between oxytocin concentrations and other variables of interest: sex, pregnancy status, median group size, degree of social access, and rates of affiliative and agonistic behaviors ($p > 0.10$). It is possible that more frequent sampling might better detect associations between oxytocin and transient social dynamics. This study provides the first data on oxytocin concentrations in black and white rhinoceroses and establishes a foundation for further investigations.

Introduction

The critically endangered black rhinoceros (*Diceros bicornis*) and the near threatened white rhinoceros (*Ceratotherium simum*) are commonly managed *ex situ* to provide important assurance populations and opportunities for breeding, reintroduction to the wild, conservation education, and research [1,2]. Physiological monitoring of rhinoceros (henceforth referred to as rhino) reproduction and wellbeing traditionally relies on steroid hormone analyses, such as progesterone, testosterone, or glucocorticoids, given their stability and robustness compared to protein hormones [3,4]. Nevertheless, protein hormones like anti-Müllerian hormone and prolactin are emerging as informative biomarkers of rhino health and biology [5,6], and oxytocin is an equally promising candidate for incorporation into the physiological toolkit [7,8].

Oxytocin, a pituitary neuropeptide hormone associated with the regulation of reproductive and social behaviors, is conserved across vertebrates [9,10]. Central oxytocin is primarily synthesized by paraventricular and supraoptic nuclei in the hypothalamus and acts within the brain to modulate social behavior and cognition [10]. In contrast, peripheral oxytocin is released into the bloodstream by the posterior pituitary and facilitates smooth muscle contractions and milk let-down during parturition and lactation [11]. Activation of the oxytocin system may involve central, peripheral, and/or coordinated release, but correlations between measures of central and peripheral oxytocin are complex and context-dependent [reviewed by [12]. Oxytocin influences reproductive and prosocial behaviors in humans such as sexual arousal and copulation [13], parental care and social bond formation [14,15],

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and social memory [16]. Increases in endogenous oxytocin concentrations have been linked to social bonding in chimpanzees (*Pan troglodytes*) [17], group composition in gorillas (*Gorilla gorilla gorilla*) [18], and daily access to a paddock in sport horses (*Equus caballus*) compared to those housed in indoor stalls [19]. In addition, oxytocin plays a role in the hypothalamic-pituitary-adrenal (HPA) axis and the mitigation of stress and anxiety [10,20], and may help regulate processes related to brain development, aging, and physiological and psychological well-being [11]. These studies support oxytocin's relationship to positive social experiences and its potential utility as an indicator of positive animal wellbeing [8], which is lacking for many zoo species including rhinos [21,22].

The present study aimed to measure monthly peripheral plasma oxytocin concentrations in black and white rhinos and to investigate associations between oxytocin concentrations and species, age, sex, pregnancy and lactation status, social management factors, and rhino social behaviors. Wild black rhinos are observed solitarily or in small transient groups [23], whereas white rhinos are more gregarious [24,25]. The natural history of black and white rhino social organization informs the management guidelines of *ex situ* populations [26]. As a result, we hypothesized that oxytocin concentrations would be higher in white rhinos than black rhinos and higher in individuals with larger group sizes and direct access to conspecifics compared to those with smaller group sizes or no social access. We also hypothesized that oxytocin concentrations would be higher in lactating than non-lactating females. An exploratory analysis was performed to test for potential associations between peripheral oxytocin concentrations and rhino behavioral frequencies. Although studies relying on infrequent oxytocin sampling often yield non-significant associations, likely due to the pulsatile nature of oxytocin secretion [27,28], establishing whether monthly sampling captures meaningful behavioral variation is an important first step for designing future, more temporally refined studies. Understanding the ways in which oxytocin concentrations might differ based on demographic and socio-environmental factors is essential for subsequent attempts to interpret differences in oxytocin concentrations related to physiological or psychological function and dysfunction.

Methods

Study animals

The study population consisted of 36 black rhinos (n = 19 male, 17 female) and 60 white rhinos (n = 20 male, 40 female) housed across 33 North American facilities (Supplementary Table 1). Ages ranged from 3 to 32 years old for black rhinos and 3–44 years old for white rhinos. Daily management and husbandry practices followed the guidelines described in the Rhinoceros Husbandry Manual [26]. Pregnancy records and parturition dates were used to identify which rhinos were pregnant (n = 2 black rhinos, 15 white rhinos) or lactating during the study period (n = 3 black rhinos, 13 white rhinos). Female rhinos have a lactation period of 15–24 months [29]. Calves begin supplementing with solid food at around two months of age and suckling frequency gradually declines over two years [30]. A rhino's median group size (i.e., 1, 2–3, 4–5, or 6 + rhinos) was calculated from the group size ranges provided by animal care staff. In addition, rhinos varied in their degree of access to conspecifics and were classified as having either no social access (none; e.g., only one rhino at the facility), proximate social access (proximate; e.g., rhinos shared a habitat border and experienced limited auditory, olfactory, visual, or tactile interaction), or direct social access (direct; e.g., rhinos directly shared habitat space). In this study, most black rhinos were housed solitarily (n = 32/36) and most white rhinos were housed socially (n = 52/60). Institutional Animal Care and Use Committee approval for this study was received from the Center for Conservation & Research of Endangered Wildlife (CREW) of the Cincinnati Zoo & Botanical Garden (22−173, 23−184), and George Mason University (1833567–1).

Sample collection

Monthly plasma samples (n = 709, 2–12 samples per rhino; Supplementary Table 1) were collected for 12 consecutive months between April 2022 and April 2024 via voluntary blood draws performed by each facility's respective veterinary and/or animal care teams. Each facility was provided collection tubes containing EDTA (Covetrus, Dublin, OH) as an anticoagulant and protease inhibitor [31,32]. Blood was collected from either the radial or metacarpal leg vein or, less often, the auricular vein. Immediately after each collection, samples were inverted, centrifuged, and the resulting supernatant transferred to 2-mL cryovials and stored at −20°C. Samples were shipped overnight on dry ice to the laboratory at CREW and stored at −20°C until extraction.

Sample extraction and analytical assay validation

Plasma samples were extracted following a proteomic reduction, alkylation, and precipitation protocol to maximize detectability of total oxytocin [33], as preliminary trials using only precipitation yielded undetectable concentrations. All extraction reagents were obtained from Thermo Fisher Scientific (Waltham, MA) or MilliporeSigma (St. Louis, MO). Each plasma sample (400 μL) was thawed and added to a 2-mL low protein binding microcentrifuge tube followed by addition of dithiothreitol (0.5 M, 20 μL). The tube was vortexed (2500 rpm, 30 s) and incubated at 37°C for 45 min. Iodoacetamide (0.5 M, 60 μL) was added and the tube was vortexed (2500 rpm, 30 s) and incubated at room temperature (15–25 °C) for 20 min. Finally, acetonitrile (95% high-performance liquid chromatography-grade acetonitrile/5% trifluoroacetic acid, 600 μL) was added and the tube was vortexed (2500 rpm, 2 min) and centrifuged (17,000 x g, 15 min). Supernatant (810 μL) was transferred to a new tube and evaporated down under a vacuum. The dried extract was reconstituted in assay buffer (300 μL), sonicated in a water bath for 10 min, vortexed (2500 rpm, 2 min), and stored at −80°C until analysis via enzyme immunoassay (EIA).

All samples were assayed neat and in duplicate using a commercially available multi-species oxytocin EIA kit (Arbor Assays, Ann Arbor, MI). The oxytocin antibody cross-reactivities were isotocin (94.3%), mesotocin (88.4%), and lys^8-vasopressin, arg^8-vasotocin, and arg^8vasopressin (< 0.15%), and assay sensitivity was reported as 17.0 pg/mL. Serially diluted, pooled samples (n = 3 black rhino, n = 4 white rhino) demonstrated a displacement curve parallel to the standard curve ($F_{1,8} = 0.30$, $p = 0.60$, Supplementary Figure 1) and linearity across dilutions ($p < 0.001$, $R^2 > 0.99$, Supplementary Figure 1; GraphPad Prism version 10.4.0, GraphPad Software, Boston, MA), indicating successful analytical validation. A spike (+200 pg/mL oxytocin standard) and recovery test yielded 96% recovery. The mean intra-assay coefficient of variation (CV) was 7.24 ± 0.17%. The inter-assay CV for control samples was 9.63%.

Behavioral observations

Rhinos were observed using 30-minute sessions of focal animal sampling (i.e., one individual at a time), and behaviors were recorded using the ZooMonitor app [34,35]. Behavioral observations were conducted over 3–5 days and occurred twice per facility, roughly six months apart between July 2022 and February 2024 to account for seasonal variation in rhino behavior and management. Each rhino was observed during regular zoo operating hours, including five morning and five afternoon sessions per visit. A total of 11 observers were trained using video recordings. The intraclass correlation coefficient for each observer was ≥ 85%. Focal animal social behaviors were classified as either affiliative or agonistic (i.e., non-contact agonistic and contact agonistic behaviors combined; Table 1) and calculated as a rate per hour by summing the total number of occurrences per observation session and

Table 1
Ethogram of social behaviors used during live observations of *ex situ* black and white rhinos. Descriptions modified from [23,24].

Category	Behavior	Description
Affiliative	Ano-genital sniff	Close or actual touch of ano-genital area with snout
	Body contact	Touching or rubbing against another individual; exclude contact agonistic and reproductive behaviors
	Climb	Place forelegs on another individual
	Head fling	Head swung up and down rapidly
	Naso-nasal sniff	Close or actual touch of nasal area with snout
	Pant	Short, wheezy, and quick breaths from chest
Contact agonistic	Horn against horn stare	Horns pressed against each other for two or more seconds
	Horn clash	Horn lowered parallel to the ground then hit against recipient's horn; duration is short and not continuous
	Horn jab	Abrupt thrust of horn toward another individual
Non-contact agonistic	Advancing steps	Direct approach at constant speed towards another individual while head carriage is low
	Chase	Run after another individual
	Charge	Rapid advance towards another individual
	Snarl	Growl or rumbling
	Snort	Nasal inhalation or exhalation
Other	Other	Any behavior not listed
	Not visible	Focal animal is not visible; recorded as a duration

dividing by the session duration in hours. Times in which rhinos were not visible during behavioral observation sessions were excluded from the analysis.

Statistical analyses

Rhino oxytocin concentrations were analyzed in R version 4.4.2 [36] using the following packages: 'lme4' [37], 'ggeffects' [38], 'car' [39], 'performance' [40], 'MuMIn' [41], 'AICcmodavg' [42]. Generalized linear mixed models (GLMM) were constructed using the Gamma distribution with log link and with monthly oxytocin concentrations as the response variable, as the Gamma distribution is ideal for the continuous, positive, right-skewed nature of the data [43]. All models included rhino and facility identification as random effects to account for repeated, unbalanced measures. A preliminary GLMM with species as a fixed effect was used to test the hypothesis that monthly plasma oxytocin concentrations are higher in white rhinos than black rhinos. Four extreme data points, each from a different rhino, were removed from subsequent analyses based on a Cook's distance greater than one [44]. Subsequent models investigated black and white rhinos separately to distinguish potential species-specific relationships.

A reference GLMM with age and sex as fixed effects was compared to candidate models that also included either lactation status (i.e., lactating or non-lactating; females only), pregnancy status (i.e., pregnant or non-pregnant; females only), median group size (i.e., 1, 2–3, 4–5, or 6 + rhinos), or degree of social access (i.e., none, proximate, or direct) as explanatory variables. Additional GLMMs were used to test the hypothesis that samples collected from lactating females would demonstrate postpartum changes in oxytocin concentrations. Lactation periods of 0–365 days and 0–730 days were considered based on typical rhino lactation periods of 15–24 months [29]; natural splines with one or two knots were tested based on preliminary data exploration. Black rhinos were excluded from lactation- and pregnancy-related models due to limited sample sizes. Finally, monthly oxytocin concentrations were paired with mean behavioral frequencies for each rhino to assess the relationship between peripheral oxytocin and rhino social behaviors. A separate exploratory GLMM was constructed for each species with monthly oxytocin concentrations as the response variable and mean affiliative and mean agonistic rates as fixed effects.

Corrected Akaike Information Criterion (AIC_c) [45] values were used to compare model fits and identify the best models for each species (i.e., lowest AIC_c value within two) [46]. The interaction between age and sex was tested but excluded from the best models based on AIC_c. Variation inflation factors (VIF) were calculated to investigate collinearity between fixed effects in each model. All VIF values were less than 3 indicating low multicollinearity among the explanatory variables in this study [47]. All model assumptions and diagnostics were checked graphically in R [40]. Marginal R-squared (R^2_m) values were calculated to assess the proportion of variance explained by the fixed effects in the best models, whereas conditional R-squared (R^2_c) values were calculated to assess the proportion of variance explained by both the fixed and random effects. Analysis of variance tables were generated using type III Wald chi-square tests to identify significant associations between oxytocin and explanatory variables. Statistical significance was set at $p < 0.05$ and potential relationships were noted at $p < 0.10$.

Results

There was no difference in plasma oxytocin concentrations between black rhinos (155 ± 4.40 pg/mL) and white rhinos (162 ± 4.56 pg/mL; $p = 0.186$). Oxytocin concentrations increased with age in black rhinos ($\chi^2 = 3.869$, df = 1, $p = 0.049$; Fig. 1a) but not white rhinos ($p = 0.181$; Fig. 1b). There were no differences detected based on sex (black rhino: $p = 0.614$; white rhino: $p = 0.602$) or pregnancy status (white rhino: $p = 0.602$).

Whereas white rhino oxytocin concentrations did not differ between lactating females (161 ± 4.46 pg/mL) and non-lactating females (158 ± 2.98 pg/mL; $p = 0.473$), changes in postpartum oxytocin concentrations were observed in lactating females from 0 to 365 days ($\chi^2 = 12.89$, df = 1, $p < 0.001$; Fig. 2a). Oxytocin concentrations generally increased until roughly 450 days postpartum before gradually declining ($\chi^2 = 5.40$, df = 2, $p = 0.067$; Fig. 2b).

Finally, oxytocin concentrations did not differ by median group size (black rhino: $p = 0.292$; white rhino: $p = 0.284$; Fig. 3a-b), degree of social access (black rhino: $p = 0.541$; white rhino: $p = 0.299$; Figure c-d), affiliative behavior (black rhino: $p = 0.582$; white rhino: $p = 0.667$; Fig. 4a-b), or agonistic behavior (black rhino: $p = 0.558$; white rhino: $p = 0.589$; Fig. 4c-d).

Discussion

This study represents the first evaluation of oxytocin concentrations in black and white rhinos. The parallel displacement curve, dilution linearity, and spike and recovery tests demonstrated successful analytical validation of the EIA, indicating it detects oxytocin in rhino plasma. Differences in management and behavioral frequencies showed no association with peripheral plasma oxytocin concentrations, possibly because the monthly sampling intervals were too infrequent to detect oxytocin fluctuations in response to dynamic social factors. Nonetheless, samples collected from lactating white rhino females demonstrated changes in oxytocin concentrations consistent with the expected duration of lactation in rhinos, serving as a biological validation of the EIA.

There was no difference in oxytocin concentrations between black and white rhinos despite the reported differences in the species' social organization. However, a species' level of sociality may relate less to circulating oxytocin concentrations and more to how the brain is organized to respond to the hormone [15,48]. Whereas known functions of oxytocin (e.g., parturition and lactation) are largely conserved across vertebrates, the locations and densities of oxytocin receptors in the brain can vary widely among even closely related species (e.g., social prairie voles, *Microtus ochrogaster*, versus solitary meadow voles, *Microtus pennsylvanicus*, and solitary montane voles, *Microtus montanus* [49–51]; social versus solitary tuco-tuco, *Ctenomys sociabilis* and *Ctenomys haigi* [52], respectively). It is possible that spikes in oxytocin concentrations mediated by social interactions act locally in the brain where receptor

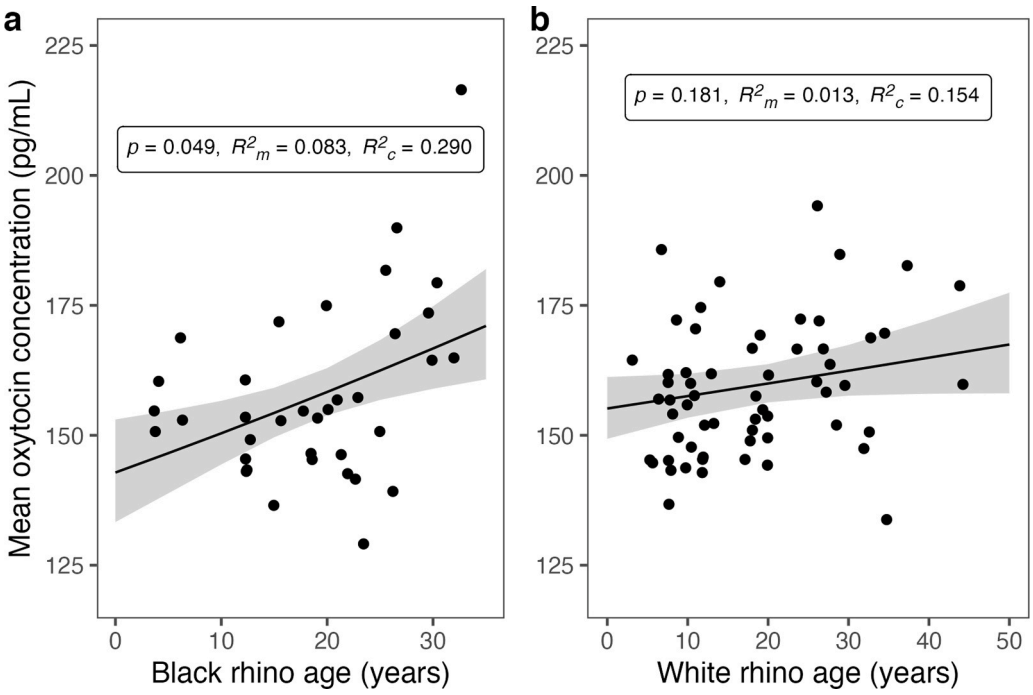

Fig. 1. Relationship between age and peripheral plasma oxytocin concentrations in black rhinos (a) and white rhinos (b). The solid line and shading indicate predicted oxytocin concentrations and 95% confidence intervals, respectively, based off monthly plasma sampling. The black circles indicate mean oxytocin concentrations per individual rather than monthly concentrations for visual clarity.

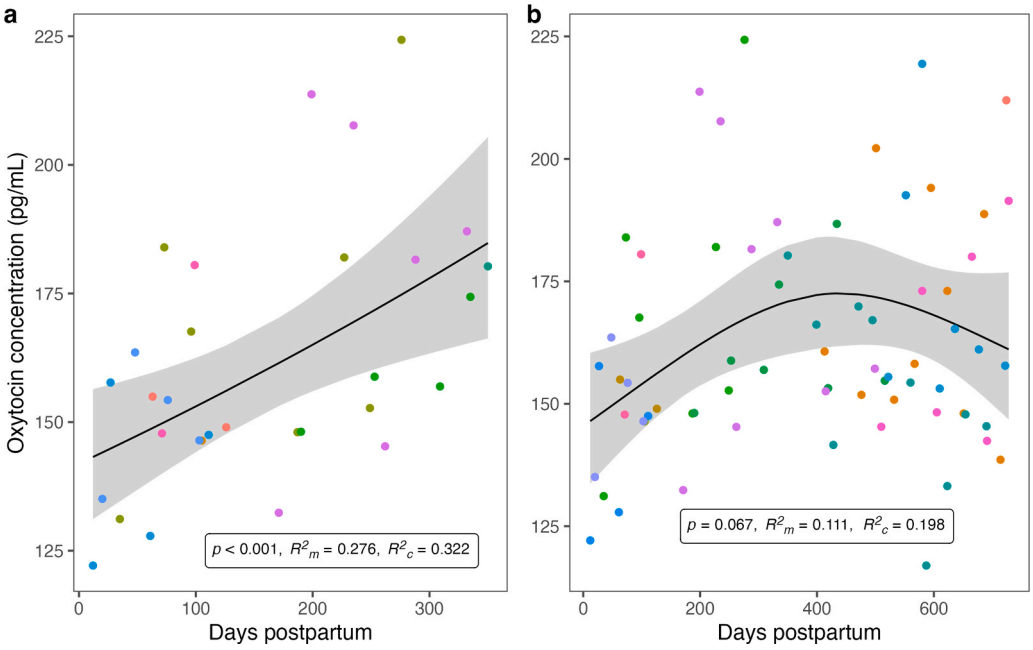

Fig. 2. Peripheral plasma oxytocin concentrations in lactating white rhino females from 0 to 365 days postpartum (a) and 0–730 days postpartum (b). Circles indicate monthly oxytocin concentrations with each color representing an individual rhino; the solid line and shading indicate predicted oxytocin concentrations and 95% confidence intervals, respectively.

density might be higher in a more social species like white rhinos compared to black rhinos, thereby reinforcing bonding and cooperation in the former but not the latter despite comparable circulating oxytocin concentrations across rhino species.

There was a weak positive association between oxytocin concentration and age, particularly in black rhinos. The positive association contrasts with patterns observed in horses [53] and lions (*Panthera leo*) [54], for which age has no relationship to oxytocin concentrations. Oxytocin in some species is elevated in juveniles, possibly to facilitate social learning during a key developmental stage, and subsequently declines with age (e.g., gorillas [18]; bottlenose dolphins, *Tursiops truncates* [55]). In the present study, exclusion of individuals under three years of age prevented the opportunity to test for a similar pattern in rhinos. Detection of late-life oxytocin patterns also was limited because few white rhinos and even fewer black rhinos over 30 years of age were sampled. Still, the weak positive relationship between oxytocin and age

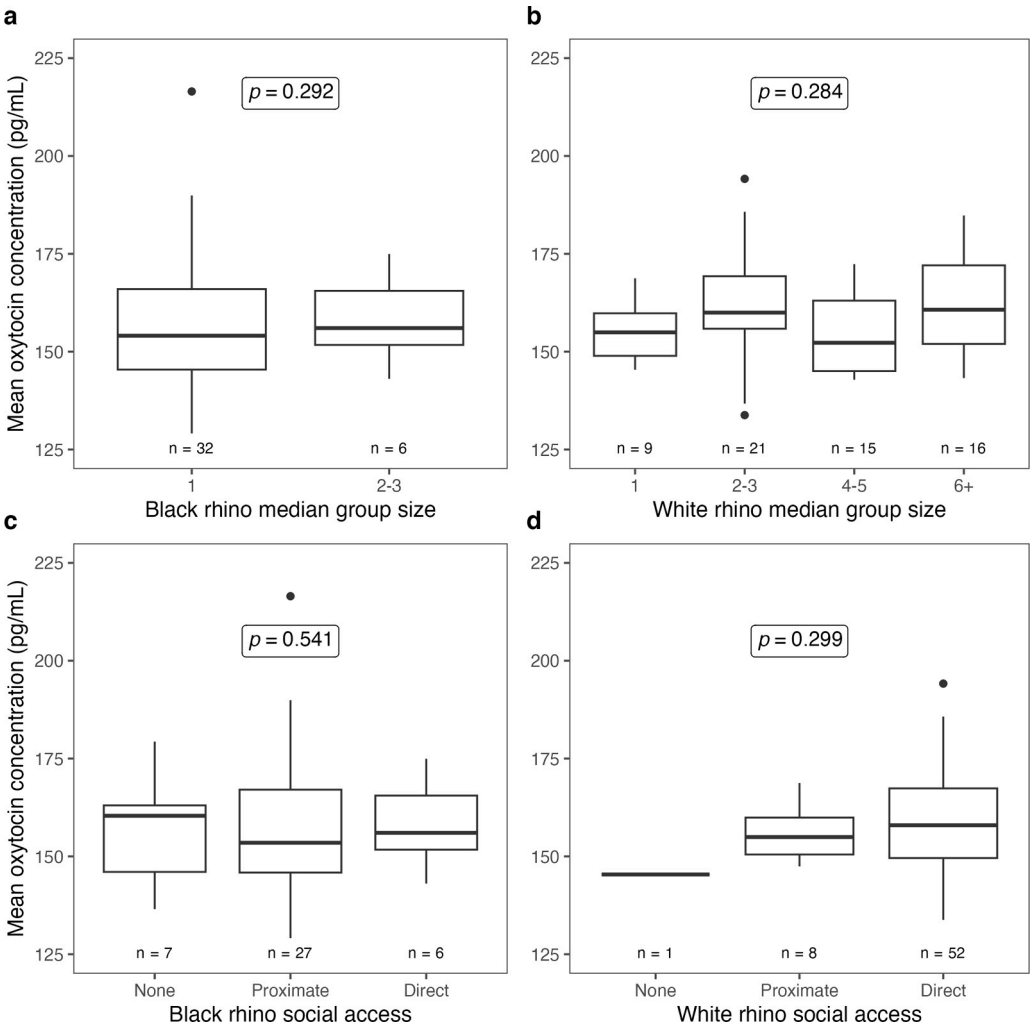

Fig. 3. Peripheral plasma oxytocin concentrations in black rhinos (left) and white rhinos (right) as they relate to median group size (a-b) and degree of social access (c-d). Mean rather than monthly oxytocin concentrations per individual were plotted for visual clarity. Sample sizes differ from the study population sample size because several individuals had samples fall into multiple categories (e.g., parturition mid-study or translocation resulting in a change in social setting).

in black rhinos is intriguing. In humans, oxytocin might have a compensatory role in offsetting cognitive decline later in life [56], associated with increases in oxytocin receptor expression in late adulthood [57,58]. Sex differences in oxytocin were not detected in black and white rhinos and are generally uncommon across taxa [59,60].

Plasma oxytocin concentrations in white rhinos increased from 0 to 365 days postpartum during lactation. However, concentrations during lactation (mean = 164 pg/mL) were only marginally higher than those of non-lactating females (mean = 159 pg/mL), and many non-lactating females demonstrated oxytocin concentrations comparable to those lactating. As a result, oxytocin concentrations did not statistically differ between lactating and non-lactating females. It is possible that more strategic sampling immediately after nursing would have revealed stronger differences [61,62]. Nonetheless, the increasing concentrations until roughly 450 days postpartum in lactating females is consistent with the typical white rhino lactation period of 15–24 months and the sustained nursing demands of the first year followed by gradual weaning [29]. A non-exclusive alternative explanation is that the increase in oxytocin is related to the duration of calf suckling bouts; in black rhinos, suckling durations increase steadily over the span of 18–20 months postpartum before declining thereafter [30]. Furthermore, the lactation-related increase and then decrease in postpartum oxytocin concentrations documented herein for white rhinos is consistent with patterns reported in other mammals (e.g., dogs, *Canis lupus familiaris* [63]; grey seals, *Halichoerus grypus* [62]; humans [64]). As the present study included incomplete oxytocin profiles from females at varying stages of lactation, future sampling across entire lactation periods will be useful for revealing more detailed changes in oxytocin concentrations during rhino lactation.

Rhino oxytocin concentrations in the present study did not vary based on group size, degree of social access, or rates of rhino social behaviors. The lack of associations potentially reflects true biological stability in circulating oxytocin concentrations across the tested social contexts. For example, plasma oxytocin concentrations in male rhesus macaques (*Macaca mulatta*) are correlated among individuals within a pair, but concentrations do not differ between pair-housed and singly housed individuals [65]. Urinary oxytocin concentrations in zoo-housed western lowland gorillas also show no relationship to group size but are higher in bachelor group males compared to mixed-sex groups and singly housed males [18]. Thus, circulating oxytocin dynamics may be particularly sensitive to more subtle contextual factors like conspecific familiarity or group composition, neither of which were examined in the present study. Other factors like estrous stage and social proximity during mating also might influence peripheral oxytocin concentrations (e.g., chacma baboons, *Papio hamadryas ursinus* [66]). Furthermore, rhinos in larger herds often form stable companion subgroups (especially white rhinos [67,68]) that may contribute to relatively consistent individual-level rates of affiliative and agonistic interactions, and

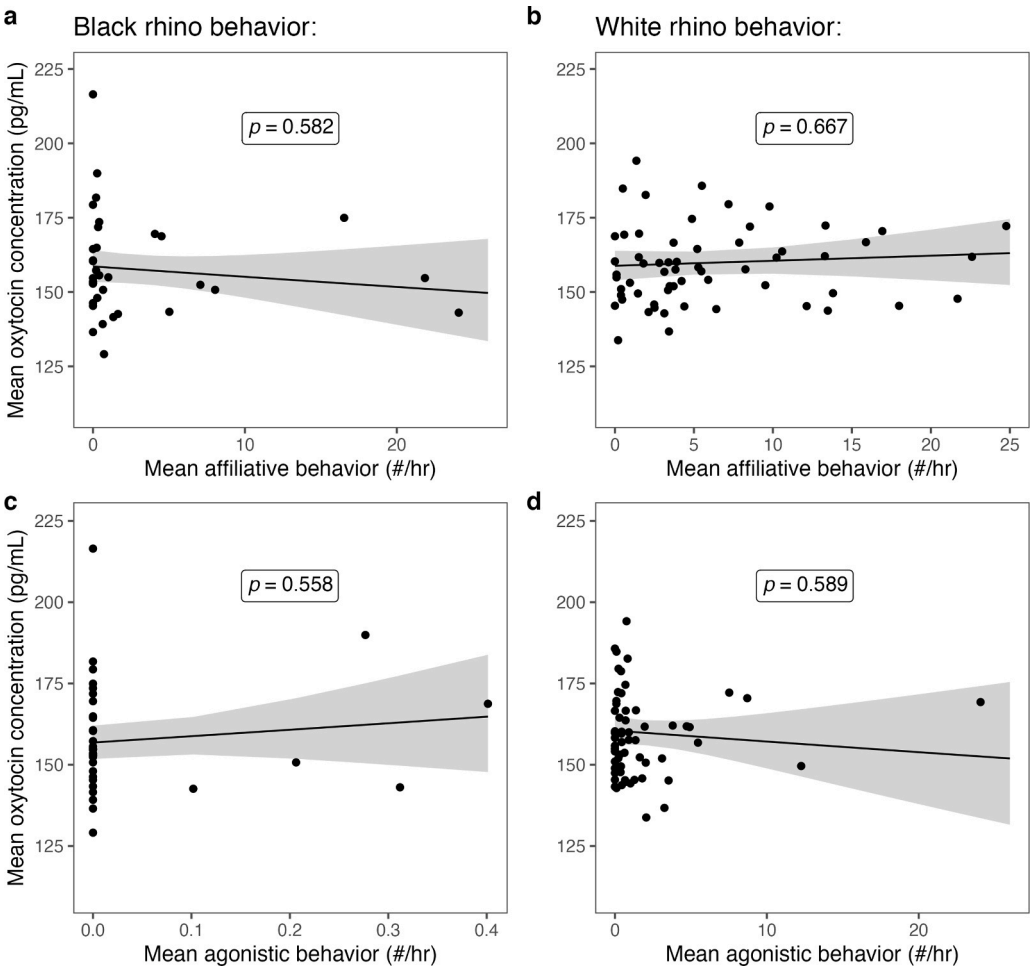

Fig. 4. Peripheral plasma oxytocin concentrations in black rhinos (left) and white rhinos (right) as they relate to affiliative behaviors (a-b) and agonistic behaviors (c-d). Solid lines and shading indicate predicted oxytocin concentrations and 95% confidence intervals, respectively, based off monthly plasma sampling. The black circles indicate mean oxytocin concentrations per individual rather than monthly concentrations for visual clarity.

subsequently consistent individual oxytocin concentrations irrespective of herd size.

Serum oxytocin concentrations in four male greater one-horned rhinos (*Rhinoceros unicornis*) were undetectable prior to intramuscular administration of synthetic oxytocin [69], and preliminary tests in the present study using only a precipitation extraction also yielded undetectable concentrations. The subsequent proteomic reduction and alkylation extraction steps used herein served to release oxytocin molecules bound to plasma proteins [33] to ensure reliable detection by the assay antibody and to eliminate interfering substances in the plasma that often result in overestimation of oxytocin concentrations [70]. Measurement of total (i.e., bound and unbound) hormone in this manner is speculated to be a better reflection of basal oxytocin concentrations compared to free hormone, which might better reflect acute secretion in its more biologically active form [71]. If true, measurement of total rather than free oxytocin in the present study might be another reason why stronger associations were not detected in relation to rhino social housing or behavior, especially given the short half-life of oxytocin (6.8 min in horses [72]; 3–6 min in humans [73]) and its pulsatile secretion [27,28]. Related to pulsatile secretion, it is possible that more frequent or strategic timing of sample collections than the monthly intervals used in this study might better detect associations between oxytocin and transient social behaviors.

In conclusion, this study represents a first effort to measure oxytocin concentrations in black and white rhinos and investigate potential relationships with various reproductive and social contexts. Results indicate the assay used in this study detected oxytocin in rhino plasma, including lactation-related changes. However, there were no differences in monthly peripheral oxytocin concentrations related to species, sex, social management conditions or behavior. Results of this exploratory study contribute to a growing body of evidence indicating that the measurement and interpretation of circulating oxytocin concentrations as a physiological measure of wellbeing is complex. Even so, the broad relevance of oxytocin to animal reproduction, health, and wellbeing highlights the importance of continued investigation.

CRediT authorship contribution statement

Drew M. Arbogast: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Elizabeth W. Freeman:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Elizabeth M. Donelan:** Writing – review & editing, Supervision, Resources, Project administration, Data curation. **Marieke K. Jones:** Writing – review & editing, Visualization, Formal analysis. **Louisa A. Rispoli:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Data curation. **Terri L. Roth:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Lara C. Metrione:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition,

Conceptualization.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.therwi.2026.100155.

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