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Fecal corticosterone concentrations and reproductive success in captive female southern white rhinoceros

Lara C. Metrione [a,b,*], John D. Harder [a]

[a] Department of Evolution, Ecology, and Organismal Biology, The Ohio State University, 318 West 12th Avenue, Columbus, OH 43210, USA
[b] South-East Zoo Alliance for Reproduction and Conservation, White Oak Conservation Center, 581705 White Oak Road, Yulee, FL 32097, USA

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ABSTRACT

Prolonged or frequent secretion of adrenal glucocorticoids in response to aversive stimuli can negatively impact reproduction. Because female southern white rhinoceros (*Ceratotherium simum simum*) reproduce poorly in captivity, we compared fecal corticosterone metabolite concentrations among parous, nulliparous, and adolescent females and examined social and physical aspects of the captive environment that might be related to differences in corticosterone metabolite concentrations. Aggression, dominance, sexual and play interactions, social group size and composition, enclosure size, and other housing characteristics were assessed though behavioral observations and review of historical and institution records. Concentrations of metabolized corticosterone in fecal samples were analyzed by enzyme immunoassay. The proportion of nulliparous females did not differ ($p > 0.05$) between subordinate and dominant animals, and subordinates did not have a higher mean fecal corticosterone concentration than dominants ($p > 0.05$). Of the behaviors examined, only the frequency of sexual play behaviors differed ($p < 0.05$) between dominants and subordinates. Average corticosterone concentrations differed ($p < 0.05$) across housing institutions but were not consistently elevated ($p > 0.05$) for females housed in most of the environmental conditions assessed. Housing with a female companion known from adolescence, however, tended to be associated ($p = 0.06$) with a lower mean corticosterone concentration than that when housing with a female companion introduced during adulthood or no female companion. Wild-caught females had a higher ($p < 0.05$) average corticosterone concentration than captive-born females. Average corticosterone concentration did not differ ($p > 0.05$) between acyclic and cycling, or nulliparous and parous females.

1. Introduction

Reproductive success is poor among captive-born female southern white rhinoceros (*Ceratotherium simum simum*) [62]. Approximately only 50% of all captive female white rhinos have reproduced [3], and only 38% of captive-born females have successfully produced young. Although there is no clear evidence of reproductive seasonality in captive white rhino males or females based on reproductive hormone patterns [6,50], irregular estrous cyclicity and completely acyclic females have been reported in several studies [6,27,50,53,59]. Another factor that may contribute to low reproductive success in white rhinos is early embryonic death [3,50,56], which has been identified by ultrasound in white [3,53], black (*Diceros bicornis*) [54], and Sumatran rhinos (*Dicerorhinus sumatrensis*) [57]. Cervical, ovarian, and uterine tumors, polyps, and cysts also have been reported [27,28], and the incidence of such pathological lesions was significantly lower in parous than in nulliparous females [27].

Stress can be described as the biological response elicited when the brain perceives a significant disturbance of homeostasis, caused by a marked or unpredictable environmental change [43,46,70]. In order to adapt to new conditions [60] and restore homeostasis [43], an animal's response to stressors includes adjustments in behavior, activation of the adrenal medulla, and the release of glucocorticoids (corticosterone and cortisol) from the adrenal cortex [17,43]. When the response to acute or chronic stress shifts sufficient resources away from other biological functions, however, deleterious effects may occur [43]. For example, chronic psychological stress can cause infertility by the actions of glucocorticoids in mammals [5]. A single significant stressor is not necessarily required; combinations of low-level stressors (exercise, diet) can synergize to compromise reproduction in *Macaca fascicularis* (70% of individuals in one study) [69]. Stressors in the captive environment of female white rhinos might activate their hypothalamic–pituitary–adrenal (HPA) axis, resulting in increased

* Corresponding author at: South-East Zoo Alliance for Reproduction and Conservation, White Oak Conservation Center, 581705 White Oak Road, Yulee, FL 32097, USA. Fax: +1 904 225 3252.
E-mail address: Lara.Metrione@sezarc.com (L.C. Metrione).

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secretion of corticotrophin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and glucocorticoids. An increase in any or all of these stress hormones could suppress reproductive function [42], often by disrupting follicle development and ovulation. Increases in ACTH and glucocorticoids can suppress luteinizing hormone (LH) [19]. Corticotrophin-releasing hormone abolishes LH pulses [55] and might decrease gonadotropin-releasing hormone (GnRH) production [12]. Even neuronal transport of GnRH peptides might be impeded in stress-sensitive animals [11].

Significant biological costs might be incurred when animals respond to frequent, various, minor stressors, such as confinement and husbandry practices [43]. For example, stress in captive cheetahs (*Acinonyx jubatus*), evidenced by higher fecal cortisol metabolites and higher corticomedullary ratios of adrenal glands relative to free-ranging cheetahs, might be associated with the high prevalence of diseases and poor reproduction in captive individuals [63]. In the wild, female and adolescent white rhinos live in groups of 2–6 [48,49,51,61], within home ranges that vary from 7 to 45 km^2 [51]. Thus, stressors for captive females might include lack of space, lack of companions, competition for clumped food resources [37,41], or social subordination.

The relationship between social status, stress, and reproduction is complex and varies considerably among species of mammals. Although chronic stress is a cost of social dominance in some species, e.g., African wild dogs (*Lycaon pictus*) [16] and dwarf mongooses (*Helogale parvula*) [16], reproduction is compromised or delayed in subordinates of other species, famously exemplified by reproductive inactivity in all but a single, dominant queen in the naked-mole rat (*Heterocephalus glaber*) [29]. A less obvious example is the positive correlation between dominance status and circulating progesterone levels following ovulation in red deer (*Cervus elaphus*), which might be caused by interference of luteal function by stress in subordinate females [21]. Black rhino females that scored higher on dominance behavior than their mate tended to be more successful breeders [10], and reproduction may be suppressed in low-ranking female white rhinos [37,41]. Interestingly, Carlstead and Brown [8] found higher fecal corticosterone metabolite variability in non-cycling compared to cycling white rhino females. If glucocorticoids are higher in subordinate females than in dominant females, then chronic social stress could be considered as a factor contributing to their reduced reproductive success. Based on this possibility, this study was designed to examine possible relationships between immunoreactive glucocorticoid metabolite (hereinafter, corticosterone) concentrations in fecal samples of captive female white rhinos and social behavior, aspects of their captive environment, and reproductive success. Behavior, reproductive success, and mean corticosterone concentration were compared between dominant and subordinate females. Fecal corticosterone concentrations also were compared between females grouped according to reproductive activity (nulliparous vs. parous; acyclic vs. cycling), place of origin (wild-caught or captive-born), enclosure and group size, number and novelty of males, the presence of a well-known companion or their mother, and residence in a natal or non-natal institution.

2. Material and methods

2.1. Historical record of housing and reproduction

The reproductive and housing history of all 45 female rhinos (13 captive-born, parous; 13 captive-born, nulliparous; 6 wild-caught, parous; 7 wild-caught, nulliparous; 6 adolescent) (Table 1) involved in any aspect of this study was evaluated. Information on enclosure and group size, the number of males available, male novelty (the male was unknown during early adolescence), the presence of the mother or a companion known during adolescence, and translocation from the natal institution was obtained from the holding institutions' written records and from the *Southern White Rhinoceros Studbook* [13,14].

2.2. Behavioral observations

Social behavior of 36 selected female white rhinos (Table 1) was recorded for 10 to 30 days (80–240 h) at each of 12 institutions during September through December 2007 and March through December 2008. Less time (<30 days) was spent at institutions that did not house mature males with the females (Birmingham, Jacksonville, Louisville) or where adolescents were the primary focus (Busch Gardens). All observations were recorded by L. Metrione during daytime hours. In addition, video recordings were made of rhinos housed in a barn during the night at the Wilds. Specific behaviors were identified according to a wild white rhino ethogram (Table 2) provided by Owen-Smith [48] and used by other investigators [37,41,62]. The frequency, context, and details of the behaviors were recorded as they occurred using continuous focal-animal and critical incident sampling [1]. Behaviors that were assessed in the final analyses included aggressive, sexual, and sexual play behaviors. In addition, keepers recorded estrus and mating for the entire duration of fecal sample collection.

Recorded behaviors were summed for every 30 min of observation for each female, providing half-hourly behavioral frequencies [37,41]. The total number of observation hours per day was, whenever possible, held constant across institutions, and the number of observation hours for each rhino within an institution was consistent and approximately equivalent between morning and afternoon sessions. To ensure the validity of using average daily frequencies for subsequent analyses, the half-hourly sums of the behavioral frequencies recorded per female during the first half of the observation period (5–15 days) per institution were compared (Spearman correlation) with those recorded during the second half of the observation period. The correlation between the first and second half-periods in frequencies of aggressive ($r = 0.856$) and sexual play ($r = 0.614$) behaviors was significant ($p < 0.05$), indicating no effect of time across the entire observation period on the recorded frequencies of those behaviors. The frequency of sexual advances by males recorded during the first half of the observation period was not correlated ($p > 0.05$, $r = 0.231$) with those in the second half, as should be expected when males change their association with females according to their sexual receptivity. As a result, an average daily frequency of sexual advances made by males to each female accurately reflects which females were courted during the observation period and which were not. Therefore, average daily frequencies for each type of behavior were calculated for each female and used in subsequent statistical analyses. Dominance was determined by calculating the percentage of the total antagonistic interactions that resulted in a "win" for each female in every possible dyad, which was then organized using dominance matrices [41,64] and dominance diagrams that compared all female–female dyads [41].

2.3. Collection of fecal and serum samples for hormone analysis

Fecal samples were collected from 31 females (Table 1). Samples ($\geqslant$50 g from fresh defecations) were collected at least once weekly from each adolescent for 1–2 years and approximately every other day (3/week) from each adult for 4 months. Schwarzenberger et al. [59] found that progesterone metabolite concentrations did not differ between the outer layer and central portion of white rhino fecal balls, thus sample location within the fecal pile or fecal ball was not considered a likely confounding

Table 1
Study population of captive female southern white rhinoceros involved in behavioral observations (September 2007–December 2008) ($n = 36$), fecal sample collection (October 2007–August 2009) ($n = 31$), and analysis of reproductive and housing history ($n = 45$).

Rhino	Institution[a]	Behavior	Samples	Rhino	Institution[a]	Behavior	Samples
Captive-born parous				*Captive-born nulliparous*			
Maggie	WOCC	X		Lucy	WOCC	X	
Gabby	Jacksonville	X	Fecal	Bonnie	LCS	X	Fecal
Julie	Wilds	X	Fecal	Kiangazi	LCS	X	Fecal
Maggie	Wilds	X	Fecal	Paddy	LCS	X	Fecal
Zenzele	Wilds	X	Fecal	Yebonga	Reid Park	X	
Bloom	LCS	X	Fecal	Dumisha	SDWAP	X	Fecal
Eliza	LCS	X	Fecal	Kiazi	SDWAP	X	Fecal
Lissa	LCS	X	Fecal	Utamu	SDWAP	X	Fecal
Taraja	LCS	X	Fecal	Taryn	WS	X	Fecal
Holly	SDWAP	X	Fecal	Jeannie	Tulsa	X	Fecal
Yvonne	Audubon	X	Fecal	Ajabu	Birmingham	X	Fecal
Laptop	Birmingham	X	Fecal	Lulu	Louisville	X	
Kendi	DAK		Fecal	Sindi	Louisville	X	
Wild-caught parous				*Wild-caught nulliparous*			
Kathy	WOCC	X	Challenge	Bertha[b]	Albuquerque		Fecal
Alice	LCS	X		Emalah	Albuquerque		Fecal
Kisiri	Busch	X		Helen	DAK		Fecal
Mlaleni	Busch	X		Jao	DAK		Fecal
Nthombi	SDWAP	X		Mashile[b]	Omaha		Fecal
Macite	Audubon	X		Marina	Omaha		Fecal
Adolescent				Mambo	Indianapolis		Fecal
Kelly	WOCC	X					
Evey	Wilds	X	Fecal				
Sally	Wilds	X	Fecal				
Dakari	Busch	X					
Lucy	Busch	X					
Kayla	DAK		Fecal				

[a] Albuquerque, Albuquerque Biological Park; Audubon, Audubon Zoo (LA); Birmingham, Birmingham Zoo; Busch, Busch Gardens (FL); DAK, Disney's Animal Kingdom; Omaha, Henry Doorly Zoo; Indianapolis, Indianapolis Zoo; Jacksonville, Jacksonville Zoo; LCS, Lion Country Safari (FL); Louisville, Louisville Zoo; Reid Park, Reid Park Zoo (AZ); SDWAP, San Diego Wild Animal Park (now San Diego Zoo Safari Park); Tulsa, Tulsa Zoo and Living Museum; WOCC, White Oak Conservation Center (FL); WS, Wildlife Safari (OR); Wilds, the Wilds (OH).
[b] Two females that reproduced in the wild but not since their capture in 1998 and 1999 were included in the nulliparous group.

factor for corticosterone metabolite concentrations in this study. All fecal samples and, later, their extract solutions or dried residues were stored at −20 °C until analysis.

Brown et al. [6] found that fecal corticoid concentrations did not differ between seasons, and so, differences in corticoid concentrations based on the time of year in which samples were collected also was not considered a confounding factor in this study. However, circulating glucocorticoid concentrations exhibit a circadian rhythm in many mammals [45]. Such variation in fecal corticosterone metabolite concentrations was not expected in rhinos. In these hind-gut fermenters, steroid metabolites accumulate in the digesta over a period of about 48 h [6] before being released in the feces, thus representing an average of circulating levels during the day. To test this assumption, corticosterone metabolites were measured in fecal samples collected 2–3 times per day for 7 days from 6 females at Lion Country Safari (LCS) and for 8 days from 4 females at San Diego Wild Animal Park (SDWAP).

2.4. ACTH challenge

Elevated corticosterone in serum or fecal samples is widely accepted as evidence of activation of a stress response in mammals, including rhinos [65]. A minimal test of this assumption is demonstration of a timely rise and fall of corticosterone following injection of ACTH, i.e., an ACTH challenge [67]. This response was previously documented in feces and serum of black rhinos [6]. In this study, an ACTH challenge was evaluated with serial blood sampling of a wild-caught, parous white rhino (Table 1). Following the protocol used by Brown et al. [6] with black rhinos, an initial baseline blood sample was collected after which an intramuscular injection of slow-release ACTH gel (2000 IU; Wedgewood Pharmacy, Swedesboro, NJ, USA) was administered. Blood samples were drawn at 1, 1.5, 2, 3, 4, 5, and 6 h after injection and were stored at −20 °C until analysis. Increasing corticosterone concentrations in serum samples collected at intervals following injection of ACTH was expected.

2.5. Extraction of steroid metabolites from fecal samples

To extract fecal steroid metabolites, 0.5 ml of deionized water and 4.5 ml of anhydrous ethanol were added to 16 × 100 mm culture tubes (Fisher Scientific, Pittsburg, PA, USA) containing 0.5 g of crushed feces. Samples were then vortex-mixed for 30 s and shaken in a horizontal position for 1 h before centrifuging for 20 min at 786*g* [40]. Fecal samples were not dried or boiled prior to extraction because Wasser et al. [67] found that the boiling and vortexing extractions produced similar recoveries (90–100%) of radioactive labeled steroids (progesterone, corticosterone, and testosterone) and their immunoreactive metabolites from both dry and wet feces. Aliquots (500 µl) of extract supernatant from every sample were dried in 12 × 75 mm culture tubes (Fisher Scientific, Pittsburg, PA, USA), and 3 ml of extract was held undiluted in reserve.

Pools of reconstituted fecal extract that were serially diluted to test for parallelism with standard curves and diluted to provide reference (control) solutions for evaluation of intra- and interassay variation were prepared by dissolving dried fecal extracts in 500 µl of 0.2 M phosphate buffered saline (PBS, containing 1.0 g/L bovine serum albumin; pH 7.0). For individual samples, 10 µl-aliquots of ethanol fecal extracts were dried at room temperature, diluted 1:40 with 400 µl of PBS added to each dried sample, and allowed to sit at room temperature for 25 h. Samples were then vortex-mixed twice and assayed immediately.

Table 2
Wild white rhino ethogram [48].

Behavior or vocalization	Purpose	Description
Snort (vocalization)	Mild "keep-away" warning	Nasal ex- or inhalation
Snarl (vocalization)	More powerful distance-increasing tool	A gruff roar, brief or rumbling, made with the mouth open, head thrust back, and ears laid back
Pant (vocalization)	Contact seeking or maintaining call	A chesty exhalation or inhalation
Hic (vocalization)	Signifies bull's intent to court	Repetitive wheezy exhalations with a throb produced at the beginning of each inhalation
Squeal (vocalization)	Signifies the actions of the bull (towards a cow) are in the context of territory boundary blocking	High pitched then falling off; may become a singing wail
Shriek (vocalization)	Attack inhibiting	Intense/shrill; ears thrust back, head thrust forward
Whine (vocalization)	Calf seeking udder or adolescents moving back toward companions	A thin, mewing tone that rises and falls in pitch
Squeak (vocalization)	Calf distress signal	Abrupt and high pitched
Gasp-puff (vocalization)	Response to sudden fright	Sudden in- or exhalation
Pinning ears back	Distance increasing display	Ears laid back, usually coupled with head thrust and snort or snarl
Advancing steps	More powerful distance- increasing effect than a snarl or snort alone	Actor steps quickly toward the recipient and simultaneously gives a snarl, snort, or shriek
Horn prod	Ritualized attack movement	Head lowered followed by upward jabbing movement
Horn clash	Gesture to repel encroachment	Horn lowered parallel to the ground then hit sideways against horn of the recipient
Charge	Intimidation display	Rapid advance
Head flings	Play invitation and indication of excitement	Head swung up and down rapidly
Presenting the side	Act of appeasement	Turning head away from other rhino
Horn against horn stare	Intimidation display	Horns of 2 bulls pressed together with heads raised and ears forward
Horn wiping	Assertion of presence/status	Sideways, twisting movements of the horn on the ground
Scraping	May be related to the deposition of scent marks	Hindlegs or forelegs dragged with nail pressed against the ground
Nasonasal meeting	Potentially for individual identification	Movements slow and relaxed eventually allowing noses to meet
Attack	To drive recipient away	Horn jabbing movements directed toward body of recipient
Fight	Opponents attempting to drive each other away	Attack gestures made by both opponents
Acceptance of tactile contact	To strengthen bonds	Expression of a close bond through non-aggressive physical contact
Urine/dung smelling	Identification	Smelling of urine or dung; may be followed by flehmen
Smelling of vagina	Estrus identification, courtship	Bull smells cow's vaginal area; may be followed by flehmen
Chin resting	Courtship	Bull rests his head on the rump or back of the cow
Mounting	Breeding	Bull straddles cow's back with forelegs while standing on hindlegs

2.6. Corticosterone enzyme immunoassay

Turner et al. [65] found approximately twice as much corticosterone as cortisol in the serum, urine, and feces of white rhinos. Therefore, we measured corticosterone concentrations in serum (ACTH challenge samples) and immunoreactive glucocorticoid metabolite (hereinafter, corticosterone) concentrations in fecal samples. Brown et al. [6] developed a glucocorticoid radioimmunoassay for serum of black rhinos and for fecal extracts from both white and black rhinos. However, this is among the first enzyme immunoassays (EIA) developed and validated for estimation of corticosterone in serum and feces of white rhinos. The assay protocol was adapted from Munro and Stabenfeldt [44] and Graham et al. [24], and the polyclonal antibody used in the assay (CJM006, provided by C. Munro, University of California, Davis, USA) was raised against corticosterone-3-carboxymethyloxime and cross-reacts with corticosterone (100.0%), desoxycorticosterone (14.25%), progesterone (2.65%), tetrahydrocorticosterone (0.90%), testosterone (0.64%), cortisol (0.23%), prednisolone (0.07%), 11-deoxycortisol (0.03%), and cortisone, estradiol 17β, and prednisone (<0.01%). Assay plates (Nunc MaxiSorp™, Roskilde, Denmark) were coated with 50 μl of antibody (1:20,000) and refrigerated overnight. After washing, 50 μl of PBS was added to each well and incubated at room temperature for 2–3.5 h. Standard, sample, or control (50 μl) was then added to each well, followed by 50 μl of corticosterone: horseradish peroxidase conjugate (HRP; 1:90,000; U.C., Davis). After shaking for 2 h at room temperature, plates were washed, and 100 μl of color-changing substrate solution (containing 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium) were added to each well. Light absorbance in the wells was measured with a 405 nm filter.

Dose-response displacement curves based on serial dilutions of pooled serum (1:1–1:64) or fecal extract (1:2–1:1000) from non-pregnant and pregnant females were parallel (Pearson correlation, $p < 0.0001$, $r > 0.99$ for all comparisons; ANCOVA test for differences in slope, $p > 0.05$) to the standard curve. The displacement curves of the pregnant and non-pregnant females were almost identical, indicating samples from both types of females could be similarly diluted. A dose-response displacement curve of pooled reconstituted fecal extract from pregnant and non-pregnant females together (1:1–1:1000) also was parallel (Pearson correlation, $p < 0.0001$, $r = 0.996$; ANCOVA test for differences in slope, $p > 0.05$) to the standard curve. Reconstituted fecal extracts were diluted 1:40, and serum samples were diluted 1:2 prior to assay. Recovery of known amounts of corticosterone (standard concentrations 0.078–10.0 ng/ml) added to pools of reconstituted fecal extract (1:40) was 132.8% (regression equation: $y = 1.4542x + 1.5096$, $r^2 = 0.999$) and added to pools of serum (1:2) was 99.5% (regression equation: $y = 1.2781x - 13.024$, $r^2 = 0.995$). Assay sensitivity was 0.078 ng/ml of standard (31.2 ng/g feces).

Pools of serum and reconstituted fecal extract were diluted in PBS to provide controls of relatively high and low hormone concentrations. The average percent of conjugate binding was 34.0% for high and 64.6% for low extract controls and 41.8% for high and 70.5% for low serum controls. Once made, fecal controls were frozen and were subsequently thawed only once before use. Serum

controls were thawed and used a maximum of 3 times. Interassay variation was 8.9% for the high extract control and 10.5% for the low extract control ($n = 51$ plates). Average intra-assay variation was 11.0% for fecal and 12.2% for serum high controls and 13.2% for fecal and 15.9% for serum low controls. Fecal sample extracts were assayed in 1 plate per female; 28 samples spanning the entire sampling period for each female were assayed, and only those samples with a coefficient of variation (C.V.) $\leqslant$ 15% between duplicate wells were used in calculating average corticosterone concentrations (ng/g ± standard error of the mean (SEM)).

2.7. Statistical analyses

Analyses of differences in behavioral frequencies between dominant and subordinate rhinos included all 32 females (adult and adolescent) observed in a group with at least 1 other female. The comparison of average daily frequency of sexual advances between dominant and subordinate females ($n = 23$) does not include adolescent females and considers only advances made by mature males. After the dominance hierarchy was determined and the females were linearly ordered, each group was divided in half, and the winning-most females were called dominant. If a group could not be divided evenly, the middle-ranked female was designated as subordinate, and thus, there was 1 fewer dominant female than subordinate females in such groups. To account for differences in average daily behavioral frequencies (given as average events per day ± SEM) that might be due to housing at different institutions rather than to an effect of dominance, 2-way analyses of variance (ANOVA) were used after applying the square-root transformation to the data. Institutions did not house equal numbers of dominant and subordinate females, so comparisons of average daily behavioral frequencies were analyzed as unbalanced, randomized block designs with sub-sampling. In Proc Mixed [58], *institution* was designated as the random effect, *dominance* as the fixed effect, and *institution* × *dominance* as the random effect of the interaction. Once the interaction was confirmed to be non-significant, it was dropped from the model.

Estrous cyclicity and pregnancy were determined for each female from progesterone or progesterone metabolite profiles and observations of estrous behavior. Baseline concentrations in progesterone and progestagen profiles were calculated using an iterative process [6,23,47] in which values that exceeded the mean + 1.3 standard deviations (SD) were excluded. A sustained ($\geqslant$12 days) rise in progesterone or progestagen concentrations followed by baseline concentrations was taken as evidence of the luteal phase of an estrous cycle [38,39]. Tests for independence (Fisher's 2-sided exact chi-square) to determine if there was a difference in the proportion of parous ($n = 26$) or cycling ($n = 12$) females between dominant and subordinate animals considered all the adult females in each housing group or companion subgroup with dominant/subordinate designations consistent with the behavioral analyses. This and subsequent analyses of parity exclude 1 nulliparous female that did not have sufficient access to a male to expect conception.

Average fecal corticosterone concentration per animal was calculated from samples collected throughout the entire sampling period because no significant difference was observed in the comparison of means calculated using samples collected from females during pregnancy compared to when they were not pregnant (see Section 3). Samples collected throughout the day at LCS and SDWAP were grouped into 3-h intervals (4 intervals from 600 to 1800 at LCS and 3 intervals from 600 to 1500 at SDWAP) for Kruskal-Wallis analysis. Spearman correlation coefficients [8–10] were used to determine whether correlations existed between rhino density and average fecal corticosterone concentrations per institution ($n = 12$), and between behavioral frequencies and average corticosterone concentrations ($n = 22$ because not all females with samples were observed). Analysis of correlation between average corticosterone concentrations and average daily frequency of sexual advances made by mature males included only 17 females because nursing females were not included and some females did not have access to mature males.

To account for differences in average fecal corticosterone concentration that might be due to housing at different institutions rather than to an effect of the variables being tested, 2-way ANOVA was used after applying the natural log transformation to the data. Comparisons of average corticosterone concentration between variables (e.g., parity) for which some but not all institutions housed females in each condition of that variable (e.g., nulliparous and parous) were analyzed as incomplete, unbalanced, randomized block designs with sub-sampling. In Proc Mixed [58], *institution* was designated as the random effect, the variable of interest as the fixed effect, and *institution* × *variable* as the random effect of the interaction. The interaction was confirmed to be non-significant in every test, and it was subsequently dropped from the model. Variables tested in this manner included dominance; place of origin; reproductive activity (estrous cyclicity and/or pregnancy); parity; and housing with a novel or familiar male, with or without the mother, with or without a familiar companion, or at the natal or non-natal institution. Proc Mixed [58] with repeated measures was used to test for a difference in average corticosterone concentration during vs. before/after pregnancy among females that were pregnant, and while housed inside vs. outside for females at the Wilds. Only 2 institutions were involved in the test for during vs. before/after pregnancy, so *institution* was treated as a fixed effect.

Comparisons of average fecal corticosterone between variables (e.g., enclosure size) for which all the rhinos at the institution could fall into only 1 condition of that variable (e.g., >0.01 km^2 or < 0.01 km^2) were analyzed as completely randomized designs with sub-sampling. In Proc Mixed [58], the variable of interest was designated as the fixed effect, and *institution within variable* was designated as the random effect. Variables tested in this manner included enclosure and group size, amount of public access, or housing with 0–1 or > 1 male. In both 2-way ANOVA designs, due to the unbalanced, incomplete sample, it was not always possible to estimate the interaction term using the mixed-model (both random and fixed effects). In such cases, all variables, including the interaction term, were treated as fixed effects. After confirming that the interaction was not significant ($p > 0.05$), the interaction term was dropped, and the mixed-model was applied as described. Differences between mean values and correlations were considered statistically significant when $p \leqslant 0.05$. *Institution* or *institution within variable* was not significant ($p > 0.05$) unless otherwise stated.

3. Results

3.1. Social behavior and reproduction

The proportion of females that gave birth (hereinafter, parity) did not differ between dominant and subordinate females within housing groups ($p = 0.68$) or within companion subgroups ($p = 0.41$). Males are typically subordinate to females, but in 2 cases of long-term, exclusive pairing, the female was subordinate and nulliparous (Yebonga at Reid Park and Jeannie at Tulsa). Copulatory behavior, including repeated mounting in all cases and intromission and ejaculation in 2 cases, was observed for 4 females, 2 of which were subordinate within their housing groups, 3 of which were subordinate within their subgroups, and 1 of which was housed with only a male. The behavior of these females and the males was consistent with previous observations of copulation be-

tween males and dominant females. Dominant and subordinate females also did not differ ($p > 0.05$) in the proportion showing evidence of estrous cyclicity or in the proportion showing evidence of any reproductive activity, including gestation, either within housing groups or within companion subgroups.

Average daily frequency of sexual play behavior differed between dominant and subordinate females. Subordinate females (housing group, $n = 17$; companion subgroup, $n = 18$) engaged in sexual play behavior more frequently (housing groups, 0.85 ± 0.21, $p = 0.013$; companion subgroups, 0.82 ± 0.20, $p = 0.021$) than dominant females (housing group, $n = 15$, 0.34 ± 0.08; companion subgroup, $n = 14$, 0.33 ± 0.08). When adolescent females were excluded from this analysis, the trend persisted within housing groups ($p = 0.08$; 0.59 ± 0.14, subordinate; 0.30 ± 0.08, dominant), but there was an interaction ($p = 0.035$) between dominance within companion subgroups and housing institution, in which sexual play differed between dominant and subordinate females depending on the institution at which they were housed. Though average daily frequency of aggressive behavior tended to differ ($p = 0.06$) across institutions, it did not differ ($p > 0.05$) between dominant and subordinate females. Dominant and subordinate females also did not differ ($p > 0.05$) in average daily frequency of sexual advances made by mature males to adults.

3.2. Corticosterone analyses

The ACTH challenge demonstrated a greater than 20-fold increase in serum corticosterone concentrations following injection of exogenous ACTH, thus confirming the biological relevance of serum concentrations from the EIA used in this study to assess stress (Fig. 1). In agreement with the findings of Turner et al. [65], no circadian pattern in corticosterone metabolites was observed at LCS ($p = 0.54$; Fig. 2) or SDWAP ($p = 0.98$; Fig. 2).

The housing institution (as a separate random effect or considered within each condition of the second variable) had a significant ($p < 0.05$) effect on average fecal corticosterone concentrations in all analyses of environmental factors, but average fecal corticosterone concentration per institution was not correlated ($p > 0.05$, $r = 0.02$) with the density of rhinos at each institution. Wild-caught females had a higher ($p = 0.034$) average fecal corticosterone concentration than captive-born females (Fig. 3). Average fecal corticosterone concentration tended ($p = 0.057$) to be lower when females were housed with a companion known from adolescence compared to housing with no female companion or a female companion that was introduced sometime during adulthood (Fig. 3). Average fecal corticosterone concentration was not affected ($p > 0.05$) by enclosure size > 0.01 km^2 (Fig. 3), by year-round public access, or by housing females in groups totaling >2 females/adolescents (Fig. 3), with novel males (Fig. 3), with >1 male, with their mother, or at their natal institution. The average corticosterone concentration in fecal samples collected from females while living in a barn at the Wilds during December–April did not differ ($p > 0.05$) from that in samples collected from the same females on pasture during May–November.

Average fecal corticosterone concentration did not differ ($p > 0.05$) between dominant and subordinate females within housing groups (Fig. 3) or within companion subgroups. Also, average corticosterone concentrations and average daily frequencies of aggression ($r = 0.13$), sexual advances made by mature males to non-nursing females ($r = -0.07$), or sexual play behaviors ($r = 0.28$) were not correlated ($p > 0.05$).

Average fecal corticosterone concentration varied among housing institutions ($p < 0.05$) in analyses of fecal corticosterone and reproductive activity and parity. Average fecal corticosterone concentration did not differ ($p > 0.05$) between acyclic and cycling females; acyclic, cycling, pregnant/lost pregnancy, and adolescent females; all non-cycling (acyclic, pregnant/lost pregnancy, adolescent) and cycling females; non-pregnant and pregnant/lost pregnancy females; or between samples collected from females during pregnancy compared to when they were not pregnant. Average fecal corticosterone concentration did not differ ($p > 0.05$) between nulliparous and parous females (Fig. 3). Among all institutions ($n = 5$) housing both parous and nulliparous females, 8/10 nulliparous females had higher average serum or fecal corticosterone concentrations than all of the parous females ($n = 9$) at the same institution. It could be argued that wild-caught females had higher corticosterone than captive-born females (nulliparous, parous, and adolescent) because all the wild-caught females were nulliparous. Indeed, 50% (8/16) of the nulliparous females, irrespective of where they were born, had higher corticosterone than any of the parous females, and only 2 wild-caught, nulliparous females had corticosterone concentrations higher than any captive-born, nulliparous females. This perspective, however, does not account for important differences in corticosterone that are attributable to the housing institution.

4. Discussion

The hypothesis that chronic social stress might contribute to reduced reproduction in subordinate females compared to dominant females was not supported by the results of this study. Nulliparous and acyclic females were not more likely to be subordinate, and

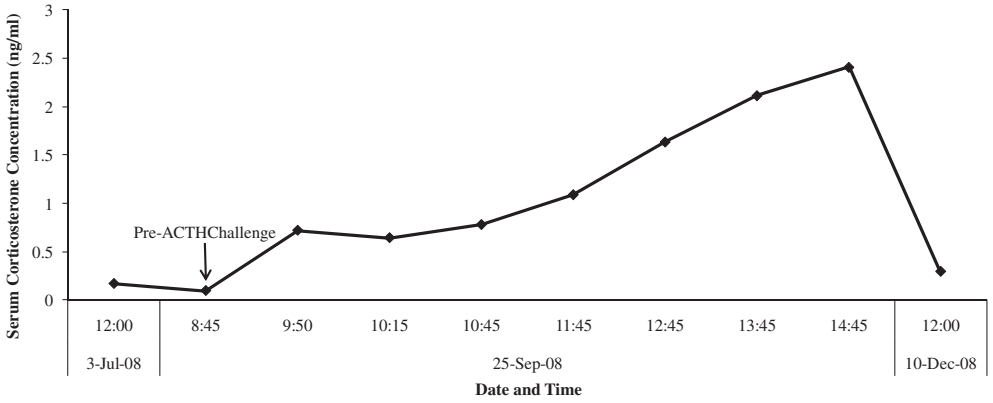

Fig. 1. Increase in corticosterone concentrations in serum samples collected from a captive female white rhino before and at 1-h intervals for 6 h following intramuscular injection of ACTH on 25 September, 2008. Baseline corticosterone concentrations are plotted for samples collected on 3 July, at pre-challenge (8:45 AM, 25 September), and on 10 December.

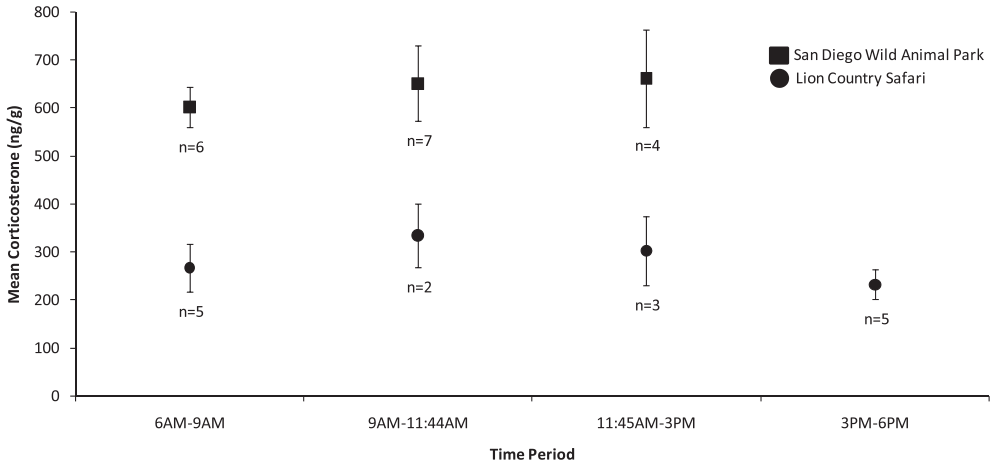

Fig. 2. Comparison of mean (±SEM) corticosterone metabolite concentrations in fecal samples collected during 3 time-periods during the day for 8 days from 4 female rhinos at San Diego Wild Animal Park and during 4 time-periods for 7 days from 6 female rhinos at Lion Country Safari. Differences in concentrations in samples collected over time were not significant ($p > 0.05$). Sample sizes on the graph indicate the number of fecal samples contributing to each mean corticosterone concentration. Slight differences in average concentration cannot be attributed to differences among females that contributed samples to a particular data point because all females were captive-born and were housed with female companions known from adolescence (the only factors for which average corticosterone concentration differs between females in this study).

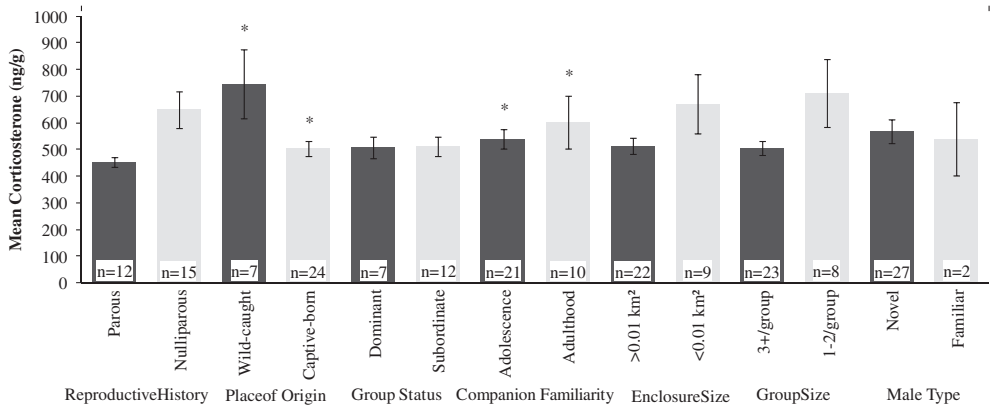

Fig. 3. Mean (±SEM) fecal corticosterone concentrations in female white rhinos compared relative to reproductive history, place of origin, social status, companion familiarity, enclosure size, group size, and male type. Corticosterone concentrations were significantly higher (∗, $p \leqslant 0.05$) in wild-caught females and in females housed with a female companion known only since adulthood or no female companion.

subordinate females did not have a higher average corticosterone concentration than dominant females. Also surprising, while average corticosterone concentration was different across institutions, place of origin and the presence of a female companion known from adolescence were the only variables that were associated with differences in mean corticosterone concentration.

4.1. Social behavior, corticosterone, and reproduction

A previous study of white rhinos suggested that subordinate females might not reproduce [37,41], and thus, subordinates might be expected to experience chronic stress and have elevated corticosterone related to their low social status. However, the results of this study, based on a larger and more diverse sample, revealed that parity was not lower in subordinate females than in dominant females. Furthermore, average corticosterone concentration did not differ between dominant and subordinate females. Similarly, no relationship was found between cortisol concentrations and dominance status or ovarian acyclicity in group-housed, mature African elephants (*Loxodonta africana*) [52]. One explanation for the similar corticosterone concentration in dominant and subordinate females in this study is that they were members of long-established groups or the females within them were familiar with each other. Long-established groups tend to have stable social hierarchies that can minimize aggression [18]. Indeed, the only behavioral difference between dominant and subordinate females observed in this study was increased sexual play behavior in the latter; the frequency of aggression did not differ. Similarly, in African wild dogs, the rate of initiation of aggressive encounters by females during the nonmating period was not affected by dominance [15]. Subordinate female rhinos lost more interactions than dominants, but they did not necessarily endure more aggressive attacks than dominants, and this might explain why the subordinate females did not have higher corticosterone than the dominant females [18]. Moreover, average daily frequencies of aggression were not correlated with average corticosterone concentrations.

Social connectedness and positive social contact can reduce the costs (high levels of stress hormones) associated with aggression and competition when living in a group [18]. For example, adult, female bongos (*Taurotragus eurycerос*) had a significantly higher frequency of sociopositive interactions when feeding in clumped situations than when feeding in dispersed feeding situations [22]. Because bongos are "concentrate selectors" [22], they encounter "contest" conditions during feeding in which access to resources is determined by rank [66]. Under clumped feeding conditions, sociopositive behaviors are a mechanism for reducing tension

[22,66]. Although white rhinos are grazers or "dispersed feeders," food in captivity is often clumped in space and time. Thus, while aggression is higher during feeding than at other times during the day [37,41], the dominance hierarchy within housing groups and companion subgroups might reduce some of the aggression during feeding. Positive contact behaviors, such as play, especially among companions, could further mitigate the stress caused by interactions with the group as a whole. It is also possible that the increased sexual play behavior on the part of the subordinate females reduces social tensions between subordinates and the other rhinos with which they are housed. It should be noted, however, that females housed exclusively with the same male since birth or early adolescence were subordinate to the male. In this particular situation, the female's subordinate status might contribute to poor reproduction, however the absence of a female companion known from adolescence also might influence reproductive success in those females.

4.2. The captive environment and corticosterone

In accord with the likely benefits of group stability and social connectedness, it appears that housing female white rhinos with a female companion known from adolescence (even if there was a period of separation between adolescence and the present) is associated with lower average fecal corticosterone concentration than that in females housed with a female companion that was introduced sometime during adulthood or with no female companion. The familiar, positive social contact of the female companion known from adolescence might reduce the likelihood of a given stressor to elicit a stress response in the focus female, or the companion's presence might reduce the duration and/or amplitude of the stress response in the focus female. The well-known female companion does not need to be the mother, as evidenced in the similarity of corticosterone concentrations in females housed with or apart from their mothers. Adolescent dispersal in the wild is associated with increased aggression at the birth of the mother's next calf [48], and the adolescent subsequently forms a companionship with another adolescent(s) or an unrelated adult female [48,49,61]. Based on these observations of wild white rhino behavior and on the tendency for corticosterone to be lower among females housed with a companion known from adolescence, managers should transport females to new institutions in pairs whenever possible, especially when relocating adolescents, as this might promote overall psychological and physiological well-being in the rhinos.

Fecal cortisol concentration was higher in male Père David's deer (*Elaphurus davidianus*) housed in small, high-density enclosures with public exposure than in free-ranging stags [34]. Similarly, fecal cortisol was higher in clouded leopards (*Neofelis nebulosa*) who had less vertical climbing space and in those on public display [68]. In contrast, although average fecal corticosterone concentration differed between institutions in our study, higher corticosterone concentrations were not associated with any particular environmental characteristics other than place of origin and the absence of a female companion known from adolescence. This suggests that environmental factors not tested in this study might account for differences between institutions. Also, white rhinos with elevated corticosterone might be individuals who are particularly sensitive to stimuli that are perceived as aversive. Considerable variability in salivary cortisol between individual Indian rhinos (*Rhinoceros unicornis*) and Asian elephants (*Elephas maximus*), possibly reflecting individual animals' abilities to cope with changes in their captive environment, was observed when a zoo opened to the public for the first time [36]. Socially-housed rhesus macaques (*Macaca mulatta*) included individuals who were behaviorally withdrawn and stress-sensitive [20]. Elevations in plasma cortisol were more sustained over time after separation events in animals characterized as highly withdrawn compared with less withdrawn animals [20]. A higher average fecal corticosterone concentration in wild-caught females compared to that in captive-born females supports the notion that wild-caught females might be stress-sensitive.

Individual rhinos showing evidence of being stress-sensitive should be considered for relocation to institutions with larger enclosures, social groups, and perhaps less public exposure, as these factors might be expected to be associated with stress in captive animals in general [7]. The ability to exert control over the termination of stressful stimuli was associated with reduced plasma ACTH in female rats [2]. Another option for the management of stress-sensitive rhinos is to provide structures within their current enclosures, such as a berm, that allow rhinos to separate themselves, at least visually, from disturbing stimuli [26]. Adequate grazing or access to abundant and widely dispersed grass hay also could give rhinos more control over their daily feeding schedule. Managers should provide logs and boulders for rubbing and manipulating as well as other enrichment activities, such as a swinging boxing bag [7], that distract the rhinos from disturbing stimuli [43] or allow them to cope with stressful stimuli through displacement behaviors [17].

4.3. Reproduction and corticosterone

Perturbations in circulating corticosterone and the other hormones of the HPA axis can impact reproduction by affecting secretion or binding of GnRH, follicle stimulating hormone (FSH), and LH, perhaps preventing ovulation [11,12,19,42,55]. However, this does not appear to be the case in white rhinos. Most nulliparous females (13/18) in this study exhibited estrous cyclicity, as evidenced in luteal-phase elevation of progestagen concentrations, and/or were in estrus based on observations of mounting and copulation [38,39]. The negative effects of altered secretory patterns of CRH, ACTH, or glucocorticoids on the hypothalamic–pituitary–gonadal axis are not evident in all species [42], and it seems that elevated corticosterone cannot account for acyclicity in white rhinos. These findings are in accord with those of Brown et al. [6] who found that corticosterone concentrations were not different between rhinos without ovarian activity and those that showed at least some ovarian activity.

Although average fecal corticosterone concentration was not statistically higher in nulliparous than in parous females, there was evidence of subtle differences across and within institutions housing both types of females. A Wilcoxon exact test, which did not account for institutional differences, indicated that nulliparous females had a higher ($p = 0.04$) average fecal corticosterone concentration than parous females. The fact that all the wild-caught females contributing fecal samples to the corticosterone analyses were nulliparous leaves room to debate which characteristic is responsible for their higher corticosterone compared to captive-born females. If there is an association between the nulliparous condition and high corticosterone instead of an association between the wild-caught condition and high corticosterone, such findings would support the possibility that corticosterone actually is higher in nulliparous than in parous females. Recall that most nulliparous females apparently experienced ovulatory cycles, and so, if further study reveals that average corticosterone concentration is higher in nulliparous than in parous females, those results would be consistent with the notion that activation of the HPA axis and chronic elevation of glucocorticoids might interfere with conception or early pregnancy.

Elevated glucocorticoid secretion mobilizes glucose, and thus, the elevated corticosterone in nulliparous females might lead to toxic levels of oxygen free radicals in the embryos when that glu-

cose is metabolized. Development of 8-cell bovine (*Bos taurus*) embryos was compromised by the addition of 4 mM glucose due to the activity of glucose 6-phosphate dehydrogenase (G6PD) [30]. Glucose 6-phosphate dehydrogenase is the rate-limiting enzyme in the pentose phosphate pathway, which generates oxygen free radicals. This might be particularly problematic for female embryos [30,33] because the gene for G6PD is *X*-linked, and the inactivation of one of the *X* chromosomes may not be completed quickly enough [25,30]. Toxic by-products from glucose metabolism may be the cause of greater female than male embryo death during early gestation among translocated white, black, and Indian rhinoceros [35]. Another possibility is that in monotocous animals, asynchrony between the sensitivity of the uterus, influenced by progesterone, and the implantation signal of the blastocyst could result in reduced fertility [31]. Linklater [35] suggested that this asynchrony might occur or be exacerbated by cortisol blocking uterine progesterone receptors, inhibiting uterine blood flow that would normally facilitate implantation. Elevated glucocorticoids also could interfere with estrogen or progesterone acting on the oviduct, slowing the speed of ovum or blastocyst transport [32]. Improper timing of oviductal transport is a cause of pregnancy loss in mares (*Equus caballus*) [4].

5. Conclusion

In conclusion, subordinate females did not have a higher average fecal corticosterone concentration than dominant females, suggesting they probably are not experiencing any more chronic social stress than dominant females. Subordinate females also were not more likely than dominant females to be nulliparous or acyclic. Average fecal corticosterone is lower in females housed with another female companion known from adolescence than in females housed with a female introduced in adulthood or with no female companion. Environmental factors other than those considered in this study need to be explored in order to understand why rhinos at some institutions have higher corticosterone than those at other institutions. Although elevated glucocorticoid levels probably are not responsible for acyclicity in white rhinos, it appears that some females, including wild-caught and possibly nulliparous females, are individuals in which the HPA axis responds more strongly to stressful stimuli. Housing females with companions known from adolescence and careful management of stress-sensitive females might result in improved reproduction in this species.

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