Zoo Biology 20:463–486 (2001)
DOI 10.1002/zoo.10028

Comparative Analysis of Gonadal and Adrenal Activity in the Black and White Rhinoceros in North America by Noninvasive Endocrine Monitoring

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Patterns of fecal reproductive steroid metabolites and adrenal corticoids were characterized for 12- to 24-month periods in black (n = 10 male, 16 female) and white (n = 6 male, 13 female) rhinoceroses at 14 institutions. All black rhinoceros females exhibited at least some ovarian cyclicity on the basis of fecal progestogen analysis (range, 2–12 cycles/yr). However, cycles often were erratic, with many being shorter (<20 days; 18% of cycles) or longer (>32 days; 21%) than the average of 26.8 ± 0.5 days (n = 104 cycles). Five females exhibited periods of acyclicity of 2–10-month duration that were unrelated to season. One complete and seven partial pregnancies were evaluated in the black rhinoceros. Fecal progestogens increased over luteal phase concentrations after 3 months of gestation. Females resumed cyclicity within 3 months postpartum, before calves were weaned (n = 5). Approximately half of white rhinoceros females (6 of 13) showed no evidence of ovarian cyclicity. Of the cycles observed, 5 were "short" (32.8 ± 1.2 days) and 24 were "long" (70.1 ± 1.6 days). Only two females cycled continuously throughout the study. One had both long (n = 9) and short (n = 2) cycles, whereas the other exhibited long cycles only (n = 5). Fecal estrogen excretion was variable, and profiles were not useful for characterizing follicular activity or diagnosing pregnancy in either species. Males of both species showed no evidence of seasonality on the basis of fecal androgen profiles. Androgen metabolite concentrations were higher ($P < 0.05$) in the black (27.6 ± 6.9 ng/g) than in the white (16.8 ± 3.1 ng/g) rhinoceros. An adrenocorticotropin hormone

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Received for publication November 30, 2000; Accepted May 23, 2001.

challenge in four black rhinoceros males demonstrated that the clearance rate of corticoid metabolites into feces was ~24 hours. Fecal corticoid concentrations did not differ between males and females, but overall means were higher in the black (41.8 ± 3.1 ng/g) than in the white (31.2 ± 1.7 ng/g) rhinoceros. In summary, fecal steroid analysis identified a number of differences in hormonal secretory dynamics between the black and white rhinoceros that may be related to differences in reproductive rates in captivity. Most black rhinoceros females exhibited some cyclic ovarian activity. In contrast, few white rhinoceroses demonstrated evidence of regular estrous cyclicity, and those females that were active had comparatively long cycles. Results also suggest that fecal corticoid concentrations reflect adrenal activity and may be species specific. Continued studies are needed to determine whether fecal corticoid measurements will be useful for understanding the cause of inconsistent gonadal activity in these two species. Because all but three (15.8%) of the white rhinoceroses evaluated in this study were less than 20 years of age compared to 73.1% (19 of 26) of the black rhinoceroses, the impact of age on reproductive and adrenal activity also needs to be evaluated further. Zoo Biol 20:463–486, 2001.

Key words: *Ceratotherium simum*; *Diceros bicornis*; fecal steroids; progestogens; estrogens; corticoids; androgens; ovarian activity, pregnancy; testicular activity; adrenal activity

INTRODUCTION

There are approximately 2,600 black (*Diceros bicornis*) and 8,500 white (*Ceratotherium simum*) rhinoceroses free-ranging in Africa [Foose, 1999]. Of the five extant species, the black rhinoceros has experienced the most severe decline in numbers during the last 15 years, with populations reduced by up to 85%. The greatest threat to the existence of the black and white rhinoceros continues to be poaching. In recent years, antipoaching efforts have stabilized numbers, yet it remains important to establish viable ex situ populations as hedges against extinction and to educate the public about the importance of species and habitat protection. Currently, there are fewer than 130 individuals of each of the two African rhinoceros species in North American institutions [Foose, 1999]. Unlike their wild counterparts, the reproductive rate of captive rhinoceroses is highly variable, with few facilities having consistently successful propagation programs.

Establishing self-sustaining populations of both African rhinoceros species requires increasing reproductive rates, while simultaneously reducing mortality. A high priority is understanding the reproductive status of the captive population and the factors that influence fecundity. Hormones drive the reproductive process, and endocrine patterns can indicate reproductive health. Remarkable progress has been made in assessing ovarian function in female African rhinoceroses, primarily through the analysis of excreted progestogen metabolites [Schwarzenberger et al., 1993, 1996, 1998; Berkeley et al., 1997; Garniera et al., 1998; Patton et al., 1999; Radcliffe et al., 1997, 2001]. A variety of assay methodologies (enzyme immunoassays and radioimmunoassays) have been used, with the majority favoring broad-spectrum antisera that cross react with a variety of pregnanes. Still, the following questions remain: 1) how consistent is cyclicity in the black and white rhinoceros; 2) what is the physiological significance of the different cycle types that have been reported in the white rhinoceros; 3) are there androgen differences between breeding and nonbreeding males; 4) do male or female rhinoceroses exhibit seasonality in gonadal or adrenal

(i.e., fecal corticoids) activity; and 5) is there a relationship between adrenal activity and reproductive status?

Therefore, a long-term study was conducted to begin generating a hormonal database in an effort to address some of these questions. The first step involved comparing individual and species differences in excreted gonadal and adrenal steroid metabolites in a subset of the North American black and white rhinoceros population. This study was only possible because of the assistance provided by the 14 collaborating North American institutions holding these two species.

METHODS

Animals and Fecal Sample Collection

Animals in this study included 10 male and 16 female black rhinoceroses at 10 zoos and six male and 13 female white rhinoceroses at five zoos (Table 1). All animals were of adult age. For the black rhinoceros, seven of the males and 14 of the females were proven breeders. For the white rhinoceros, three of the males and three of the females were proven breeders. In general, black rhinoceros females were housed separately, whereas white rhinoceros females were housed together. For both species, males and females were put together only during periods of attempted breeding. Most zoos with males conducted at least some breeding introductions during the course of the study. At all facilities, males and females were in close proximity and had visual and/or olfactory contact.

Individual fecal samples (10–50 g) were collected three to seven times weekly for females and once weekly for males for 12- to 24-month periods. After collection, samples were frozen and stored at –20°C until analysis. To identify individual fecal samples from white rhinoceroses housed in groups, the following markers were tested in preliminary trials: 1) sunflower seeds mixed with grain; 2) biodegradable barrier tape torn into small pieces added to grain; 3) cake-decorating dye (several colors of

TABLE 1. Summary of institutions with black and white rhinoceroses participating in the study

	Black rhinoceros			White rhinoceros		
	N	Age (yr)[a]		N	Age (yr)[a]	
Institution	(M/F)	Males	Females	(M/F)	Males	Females
Cameron Park Zoo	—	—	—	1/2	10	25/27
Caldwell Zoo	2/1	11/11	11	—	—	—
Cincinnati Zoo	0/1	—	29	—	—	—
Dallas Zoo	2/1	3/15	7	—	—	—
El Coyote	1/3	25	8/9/20	—	—	—
Fort Worth Zoo	1/2	8	8/21	1/1	27	27
Fossil Rim	1/2	12	7/20	0/1	—	34
Knoxville Zoo	—	—	—	0/1	—	20
Milwaukee Zoo	1/1	12	15	—	—	—
North Carolina Zoo	—	—	—	2/2	27/27	27/29
Riverbanks Zoo	1/1	11	9	—	—	—
San Antonio Zoo	1/2	28	12/28	—	—	—
White Oak	0/2		15/18	—	—	—
The Wilds	—	—	—	2/6	11/21	11/26/26/26/28/34
Total	10/16	13.6 ± 2.4	14.8 ± 1.8	6/13	20.5 ± 8.4	26.2 ± 1.6

[a]Age (or estimated age) as of 1997.

concentrated paste) placed into the middle of apples; and 4) art glitter (crystal; Sulyn Industries, Coral Springs, FL) mixed with grain. Unfortunately, none of the markers was considered appropriate for this study (see Results); thus, samples from group-housed animals were collected only from animals housed alone at night or after direct observation of defecation.

At facilities where male:female introductions were conducted (Milwaukee Zoo, Fort Worth Zoo, White Oak Conservation Center, El Coyote, Caldwell Zoo, North Carolina Zoo, Knoxville Zoo), breeding behavior and parturition dates were recorded.

Fecal Steroid Extraction

Frozen feces were lyophilized, pulverized, and the fecal powder was stored at –20°C until steroid extraction. A 0.2-g aliquot of well-mixed powder was boiled twice in 5 mL of 90% ethanol:10% distilled water for 20 minutes. After centrifuging at 500*g* for 10 minutes, supernatants were recovered, dried, and redissolved in 1 mL methanol. Extractants were vortexed (1 minute) and then sonicated for 30 seconds. Samples were diluted (1:40 for estradiol; 1:800– 1:80,000 for progestogens; 1:10 for androgens; 1:10 for corticoids) in phosphate-buffered saline (0.01 mol/L $NaPO_4$, 0.14 mol/L NaCl, 0.5% bovine serum albumin [BSA], 0.01% NaN_3) before radioimmunoassay (RIA). Extraction recovery efficiency was >85% for all steroids.

Radioimmunoassays

The progesterone RIA relied on a monoclonal antibody produced against 4-pregnen-11-ol-3, 20-dione hemisuccinate:BSA (provided by Dr. Jan Roser, University of California, Davis), a ^{125}I-progesterone tracer (ICN Biomedicals, Inc., Costa Mesa, CA) and progesterone standards. Assay sensitivity, based on 90% of maximum binding, was 3 pg/mL. Two RIAs were tested for characterizing follicular activity. Assay selection was based on 1) dilution curve analysis using the method that detected the most steroid metabolite mass for each species; and 2) metabolic excretory byproducts that were known to predominate [Hindle et al., 1992; Schwarzenberger et al., 1998]. A total estrogen double-antibody ^{125}I RIA (ICN Biomedicals) was used to quantify follicular activity in the black rhinoceros. An estradiol RIA that used an antibody raised in rabbits against estradiol-17β 6-*o*-carboxy-methyloxime:bovine serum albumin (provided by Dr. S. Wasser, Seattle, WA), a ^{3}H-estradiol tracer, and estradiol standards were used for the white rhinoceros. Assay sensitivities were 2 pg/mL and 5 pg/mL for the total estrogen and estradiol assays, respectively. A double-antibody ^{125}I RIA for testosterone (ICN Biomedicals) was used to quantify immunoactive androgen metabolites. Assay sensitivity was 0.5 ng/mL.

Two RIAs were tested for assessing corticoid (i.e., adrenal) activity in weekly samples from all individuals in the study. One was a solid-phase ^{125}I RIA (Coat-A-Count; Diagnostic Products Corporation [DPC], Los Angeles, CA), and the other was a double-antibody ^{125}I RIA for corticosterone (ICN Biomedicals). Assay sensitivities were 2.5 and 25 ng/mL, respectively. The biological validity of these assays was determined after an adrenocorticotropin (ACTH) challenge in four black rhinoceros males, and by dilution curve analysis in both species. Daily fecal samples were collected for up to 4 days before ACTH administration to establish baseline fecal corticoid metabolite values. A slow-release ACTH gel (800 IU custom preparation; Medicine Shop, Front Royal, VA) was administered as two i.m. injections (400 IU in 5 mL, given 5 minutes apart) by a Telinject system (Telinject U.S.A., Inc., Santa Clarita,

CA) (n = 2 males) or as a single injection via stick pole (n = 2 males). Daily fecal samples were collected for up to 1 week after ACTH and stored at –20°C until analysis. Only the corticosterone RIA measured detectable levels of corticoid immunoactivity in fecal extracts, and an increase in corticoids after ACTH. Thus, this assay was used to evaluate adrenal function in both species. In two animals, blood also was collected during the ACTH challenge. Samples were collected immediately before ACTH and 1 hour after injection. Serum cortisol was measured using the DPC cortisol RIA.

Each assay was validated for rhinoceros fecal extracts or serum by demonstrating 1) parallelism between serial dilutions of sample and the standard curve; and 2) significant mass recovery of exogenous steroid added to samples before analysis. For all assays, intra- and interassay coefficients of variation were <15%.

High Performance Liquid Chromatography

The number and relative proportions of corticoid and androgen metabolites in black and white rhinoceros fecal extracts were determined using reverse-phase high-performance liquid chromatography (HPLC) (Microsorb C-18 Column; Rainin, Woburn, MA). Dried ethanol supernatants from pooled fecal extracts were reconstituted in 0.5 mL phosphate buffer (pH 5.0), passed through a C-18 matrix column (Spice Cartridge; Rainin), and eluted with 5 mL 80% methanol to remove contaminants. One hundred microliters containing ~10,000 dpm each of ^{3}H-cortisol, ^{3}H-corticosterone, and ^{3}H-desoxycorticosterone (for corticoid determination) or ^{3}H-aldosterone, ^{3}H-dihydrotestosterone (DHT), ^{3}H-testosterone, ^{3}H-androstanediol, and ^{3}H-androstenedione (for androgen determination) were added to the filtered fecal extracts and coeluted. Samples were evaporated to dryness, reconstituted with 300 μL methanol, and 55 μL were injected onto the column and eluted using a gradient of 20–100% methanol:distilled water (corticoids) or 45% isocratic acetonitrile:water (androgens) during 80 minutes. One-milliliter fractions were collected (1 mL/minute flow rate). One hundred microliters of each fraction were removed for radioactivity determination and the remaining 900 μL were dried down and reconstituted in 125 μL phosphate buffer (pH 7.4) for quantification of corticoid metabolite immunoreactivity.

Data Analysis

Mean data are presented as ± SEM. Definition of the estrous cycle was based on fecal progestogen profiles. For each female, a nonpregnant baseline progestogen value was calculated using an iterative process in which values that exceeded the mean plus 1.5 standard deviations (SD) were excluded. The average was then recalculated and the elimination process was repeated until no values exceeded the mean plus 1.5 SD [Brown et al., 1994b]. Onset of the luteal phase was defined as the first point after values increased above the baseline by 50% and remained elevated for at least 2 consecutive weeks. The end of the luteal phase was defined as the first of two consecutive values that returned to baseline concentrations. Estrous cycle length was calculated as the beginning of one luteal phase to the beginning of the next. Anestrus was defined as an interluteal period exceeding twice the length of a normal follicular phase [10 days for black rhinoceros: Hindle et al., 1992; 30 days for white rhinoceros: Schwarzenberger et al., 1998; Patton et al., 1999]. In some cases, subjective observations were used to distinguish differences between cyclic changes in progestogen excretion and random fluctuations in the data. When samples were collected at

least every other day, one point peaks were ignored. For the black rhinoceros, the cycle length was considered normal if it was between 20 and 32 days (i.e., ±6 of the mean). For the white rhinoceros, cycles were categorized as short if they were <45 days and long if they were >50 days in duration.

Gender, age, and species differences in fecal steroid concentrations were determined by averaging individual means and compared using *t*-tests. For fecal androgens and corticoids, weekly means were calculated for each individual, and seasonal effects were evaluated by analysis of variance. Fecal corticoids were averaged during times of cyclicity versus anestrus for each female black rhinoceros, and between cycling versus noncycling white rhinoceros females. Means were calculated for the two reproductive states and compared by *t*-tests. Relationships between fecal corticoid and progestogen or androgen concentrations were determined by regression analyses.

RESULTS

Fecal Marker Study

Sunflower seeds were excreted intact, but often were consumed by birds before samples could be collected. Biodegradable barrier tape could be identified in individual samples, but was not palatable and the animals learned to disperse it by blowing on the feed. Cake-decorating dye, although consumed readily, was messy and even when fed in large quantities, the color change was subtle and samples were not easily identified. Art glitter was easily fed and detectable in feces; however, concern over feeding this synthetic product for months discouraged its use for this study.

Black Rhinoceros Females

All study females showed at least some evidence of ovarian cyclicity. There was no seasonality in ovarian activity because cycles were observed each month of the year. The average estrous cycle length for all females was 26.8 ± 0.5 days (range, 14–60 days; n = 104 cycles). One singleton female cycled regularly during a 2-year period (Fig. 1a). Others exhibited more erratic patterns, with 18% of cycles classified as short (<20 days) and 21% classified as long (>32 days) (Fig. 1b,c). Anestrous periods, ranging from as short as 2 to as long as 10 consecutive months, were observed in 10 of 16 females; however, five of these episodes occurred postpartum.

Overall baseline concentrations of fecal progestogens averaged 1.43 ± 0.41 μg/g, with peak luteal phase concentrations ranging from approximately 2 to 20 μg/g. The follicular phase (i.e., when progestogen concentrations were at baseline) generally lasted 2–5 days. Fecal total estrogen metabolite concentrations in black rhinoceros females fluctuated markedly and were not useful for characterizing follicular activity, even when samples were collected every day. Total estrogen surges were observed during the follicular phase (Fig. 2a); however, they also occurred commonly at other times during the cycle (Fig. 2a,b). Overall mean total estrogen metabolite concentrations averaged 72.6 ± 10.2 ng/g, with peak concentrations reaching as high as 300 ng/g.

Three females bred multiple times (n = 3, 5, 9 breedings/female) without conceiving (e.g., Fig. 1b). Three others that bred fewer times (n = 1, 1, 2) did become pregnant. One complete and seven partial pregnancies were monitored during the study. Five of the females were pregnant before study onset and gave birth during the evaluation period. Most breedings occurred when fecal progestogen concentrations were low. After presumed ovulation, progestogen concentrations increased and

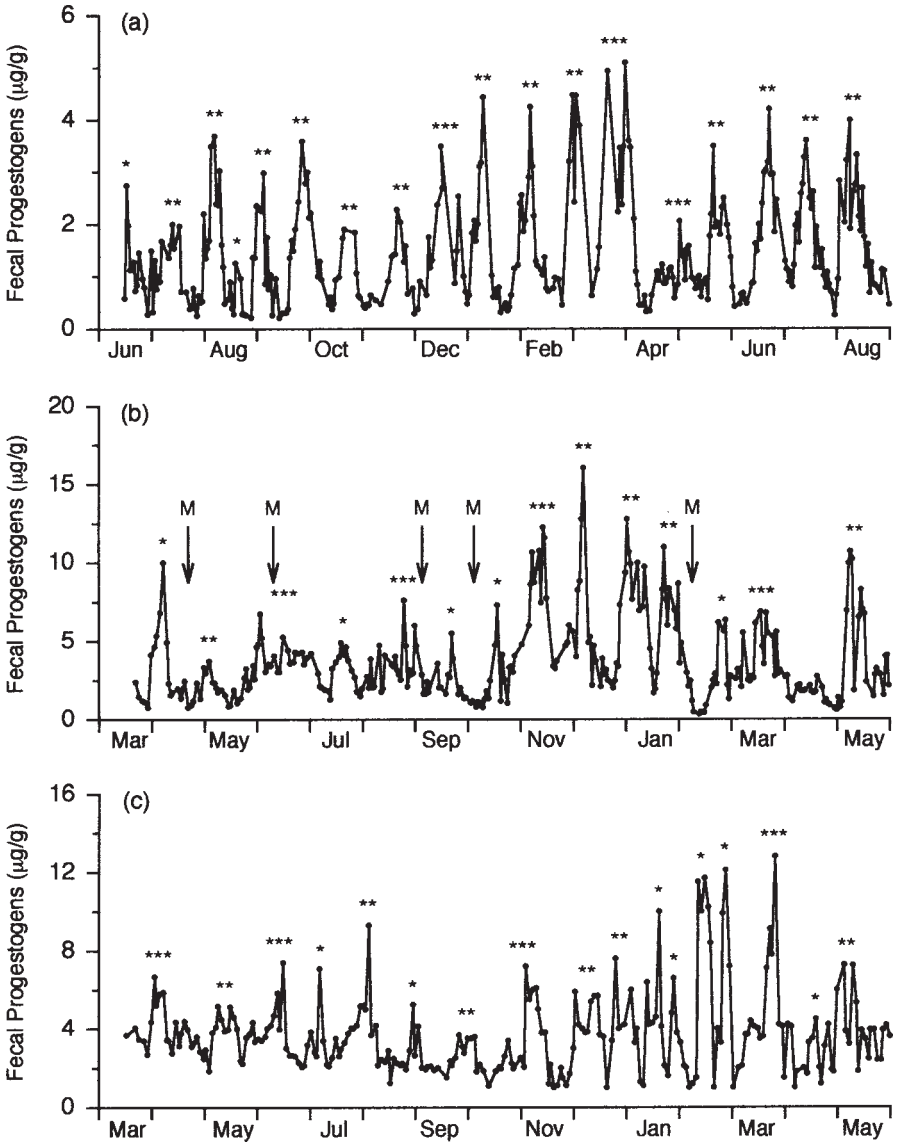

Fig. 1. Individual profiles of fecal progestogen concentrations in three representative black rhinoceros females. Reproductive cycles are designated by an asterisk (*short, <20 days; **normal, 20–30 days; ***long, >30 days). Behavioral observations were conducted only on the female in **b**. This female mated (M) multiple times without apparent conception.

remained at luteal phase levels until ~3 months of gestation, when concentrations increased markedly (Fig. 3a). As during the estrous cycle, fecal estrogens fluctuated throughout gestation and were noninformative (Fig. 3b). Fecal corticoid concentrations were relatively stable throughout gestation, except for a slight increase near parturition (Fig. 3c). Five females were evaluated for several months postpartum (Fig. 4, Fig. 5a). All demonstrated reinitiation of estrous cyclicity within 3 months of parturition, before calves were weaned (Fig. 4). One of these (Fig. 4c) appeared to

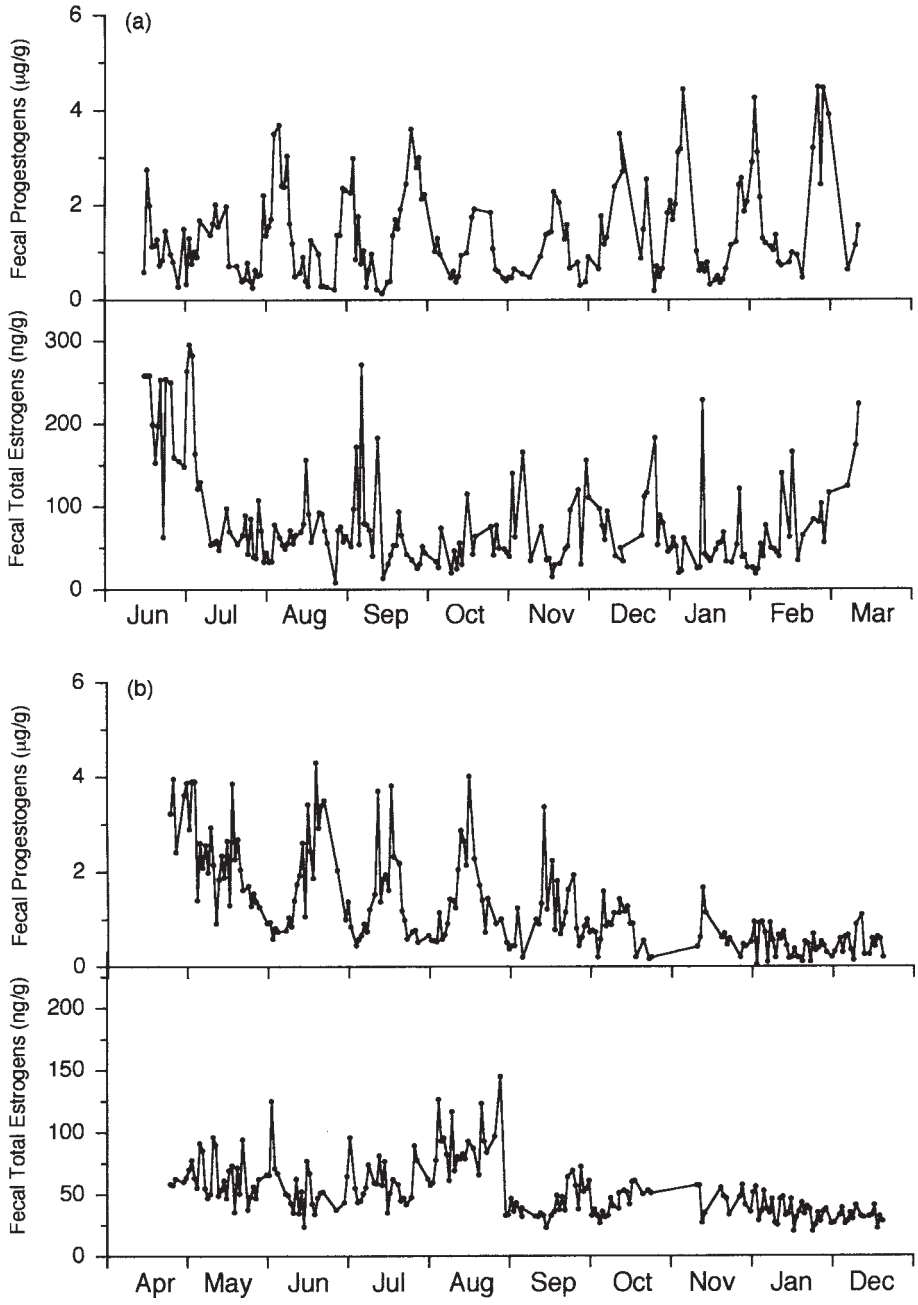

Fig. 2. **a, b:** Individual profiles of fecal progestogen and total estrogen concentrations in two black rhinoceros females.

have ovulated within a month of parturition, after which ovarian activity was suppressed for 6 months before cyclicity resumed. Another exhibited a postpartum luteal phase approximately 45 days postpartum, but remained acyclic through the subsequent 10-month period (data not shown). Two females were evaluated for several

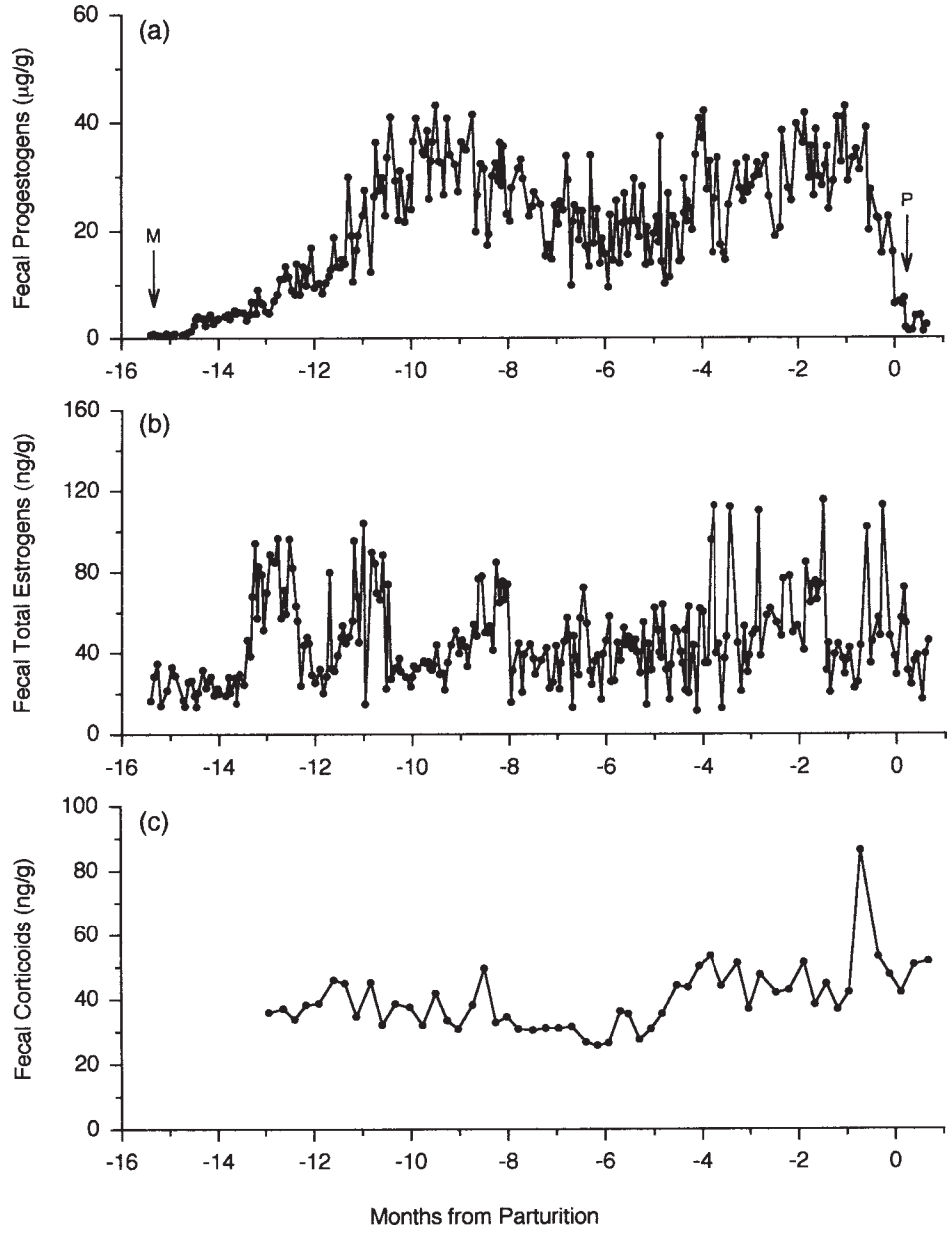

Fig. 3. Fecal progestogen (**a**), total estrogen (**b**), and corticoid (**c**) concentrations in an individual black rhinoceros female throughout gestation. Data are aligned to the time of parturition. M, observed mating; P, parturition.

months before conception (Fig. 5). One gave birth shortly after study onset (Fig. 5a). A 3-month anestrus followed before cyclic activity resumed, but then ovarian activity ceased for several months before conception occurred. The other female showed little evidence of estrous cyclicity for the first 9 months of the study, but then exhibited three cycles that led up to conception (Fig. 5b).

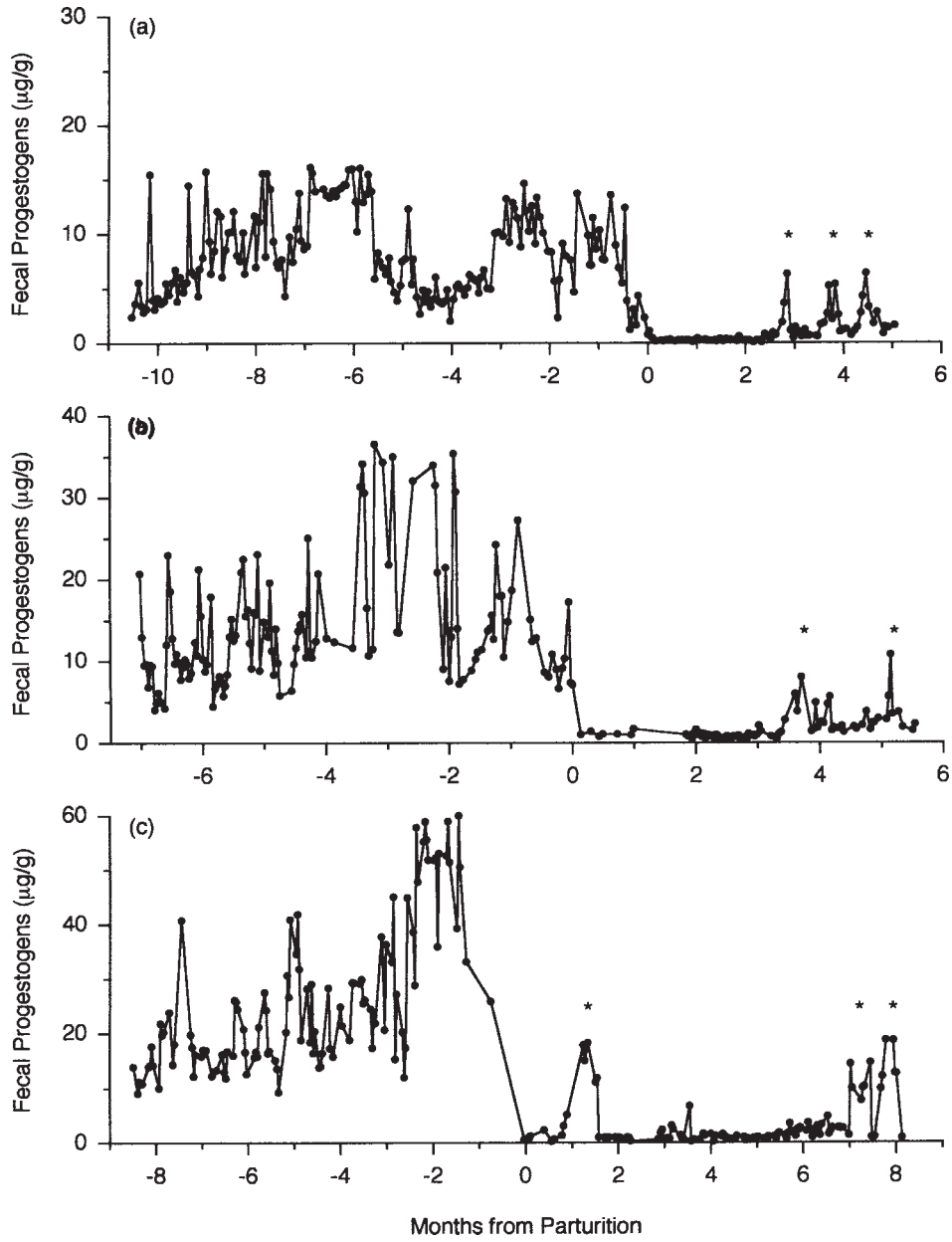

Fig. 4. Fecal progestogen concentrations in three black rhinoceros females monitored postpartum. All females nursed their calves throughout the evaluation period. Data are aligned to the time of parturition. Reproductive cycles are designated by an asterisk.

White Rhinoceros Females

Half of the females evaluated (6 of 13) showed no evidence of ovarian activity during the study period (Fig. 6a). The other seven exhibited some luteal activity, but only two cycled continuously (Fig. 6b,c). The average age of white rhinoceros fe-

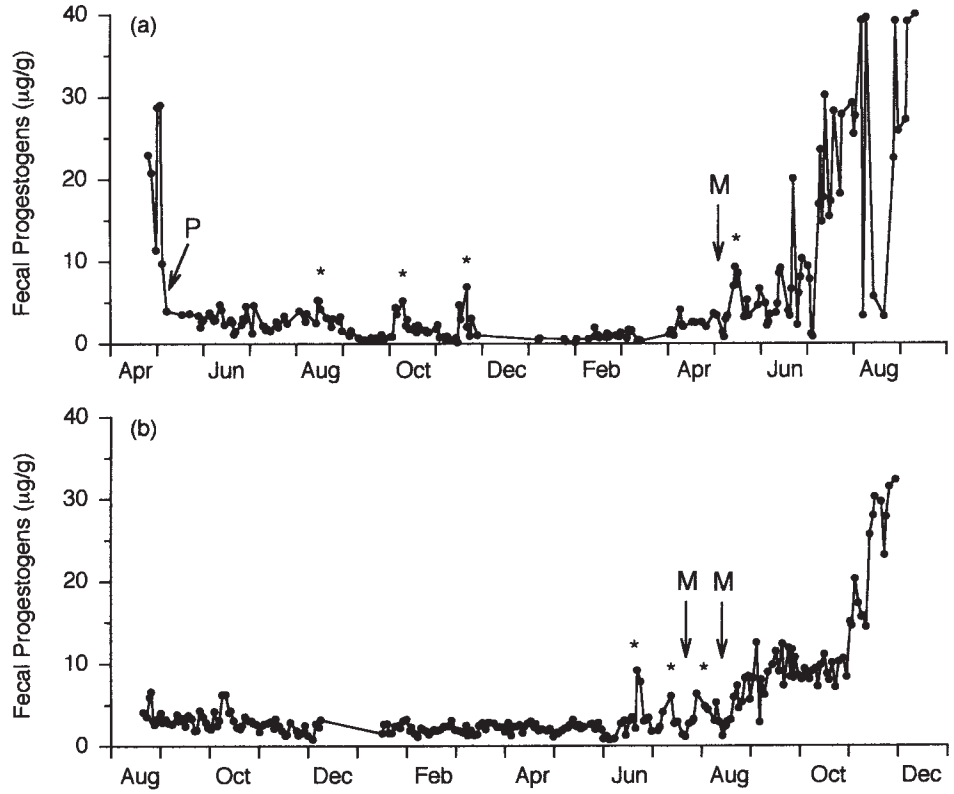

Fig. 5. Fecal progestogen concentrations in two black rhinoceros females. The female in **a** was pregnant at study onset and then conceived again. The female in **b** conceived during the last third of the study. Reproductive cycles are designated by an asterisk. M, observed mating; P, parturition.

males was greater ($P < 0.05$) than that for the black rhinoceros, and only two females were less than 25 years of age (Table 1). However, there was no age difference ($P > 0.05$) between cycling (26.9 ± 1.6 years) and noncycling (25.3 ± 3.1 years) females. Of the 29 cycles observed, 5 were categorized as "short" (32.8 ± 1.2 days) and 24 were "long" (70.1 ± 1.6 days). Of the two females that cycled year round, one had both long (n = 9) and short (n = 2) cycles (Fig. 6b). The other female exhibited only long cycles (n = 7) (Fig. 6c). These two females were 27 and 29 years of age, respectively. Of the two youngest females, one (20 years old) exhibited two short cycles and the other (11 years old) was acyclic. The follicular phase was variable and lasted from 2 to 21 days.

Average baseline fecal progestogen concentrations were 1.22 ± 0.41 μg/g, with peak luteal phase concentrations ranging from 3 to 24 μg/g. These values did not differ ($P > 0.05$) from the black rhinoceros. Breeding was observed in three females (n = 1, 4, 6 breedings/female), but no calves were produced. Two females housed together appeared to cycle synchronously (Fig. 7). Both exhibited a period of ovarian inactivity from February through June followed by three long, one short, and then one long cycle. The only difference between these two females was that overall progestogen concentrations were higher in one (Fig. 7b) than in the other (Fig. 7a).

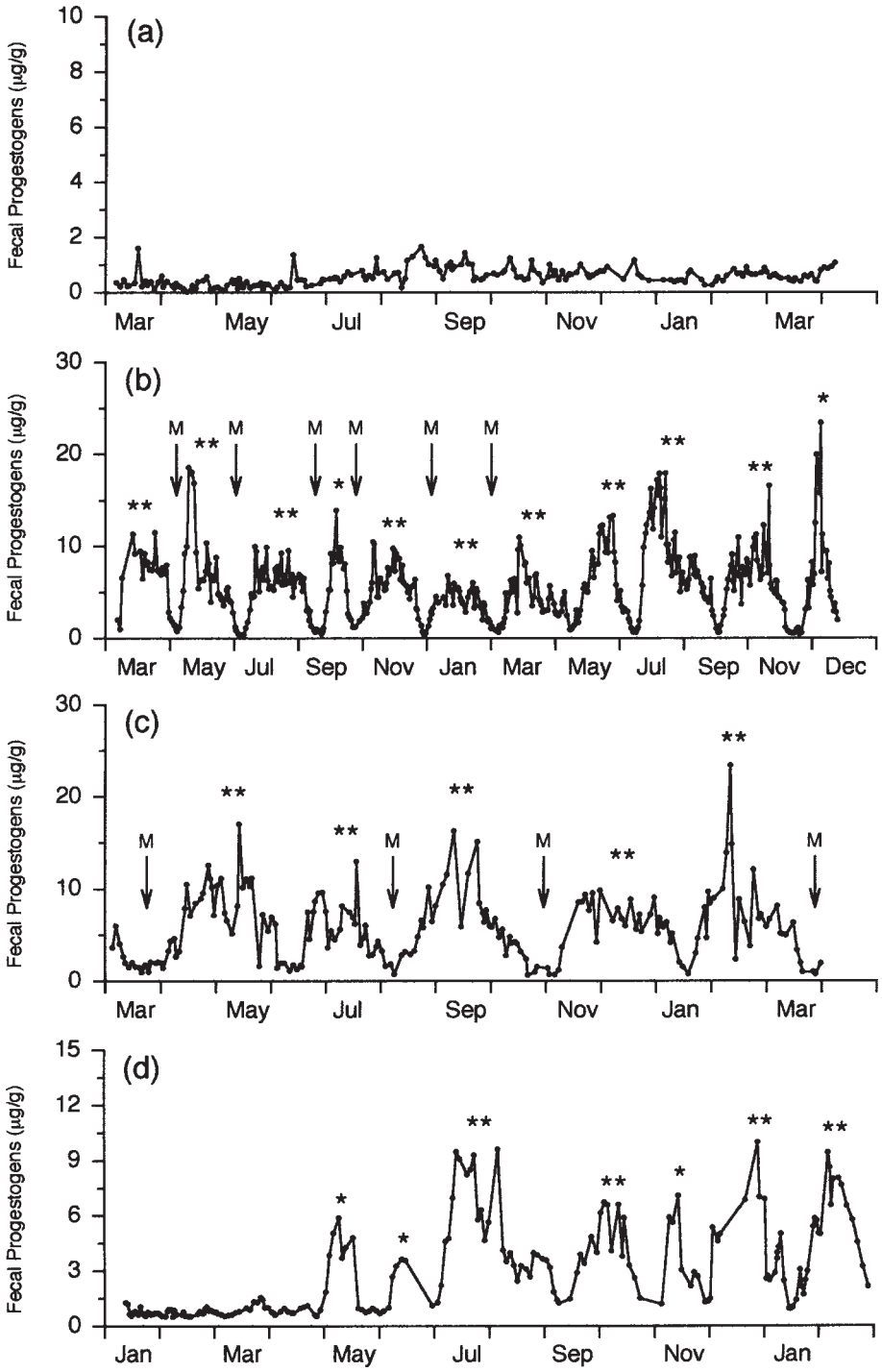

Fig. 6. Individual profiles of fecal progestogen concentrations in four white rhinoceros females exhibiting cyclic and noncyclic reproductive patterns. Females in **a**, **c**, and **d** were evaluated for 13–14 months; the female in **b** was evaluated for 22 months. Single asterisks represent short cycles, whereas double asterisks represent long cycles. Behavioral observations were conducted on females in **b** and **c**. M, observed mating.

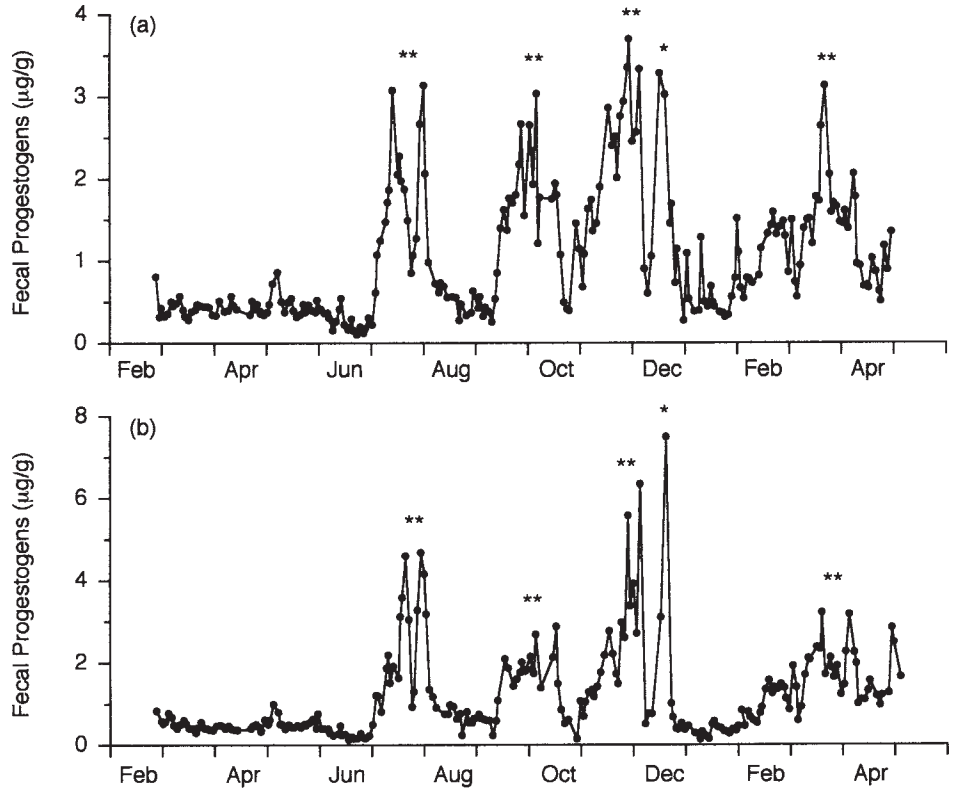

Fig. 7. **a, b:** Fecal progesterone concentrations in two white rhinoceroses housed at the same facility exhibiting apparent reproductive cycle synchrony. Single asterisks represent short cycles, whereas double asterisks represent long cycles.

There was no obvious explanation for this phenomenon other than keeper observations that the two females appeared to be particularly "bonded."

Fecal estradiol metabolite concentrations in white rhinoceros females fluctuated randomly throughout the cycle, similarly to the black rhinoceros, and were not useful for characterizing follicular activity. Estradiol surges preceded luteal phase progestogen increases in a few cycles, but peaks occurring at other times of the cycle also were common (Fig. 8). Overall mean estrogen metabolite concentrations averaged 92.6 ± 6.1 ng/g, with fluctuations occasionally reaching 200 ng/g.

Black and White Rhinoceros Testicular Activity

In both the black and white rhinoceros, androgen immunoactivity in HPLC-purified fecal extracts was associated with a peak that corresponded to the testosterone reference tracer (Fig. 9a,b). In the white rhinoceros, five additional immunoactive peaks also were identified, four of which eluted with aldosterone, androstanediol, androstenedione, and DHT, but these were of much smaller mass. The fifth metabolite peak did not coelute with any of the reference tracers, but also was of smaller mass compared to testosterone. In the black rhinoceros, a second immunoactive peak of equal mass to that coeluting with testosterone was detected. This metabolite did not coelute with any of the reference tracers. In contrast to the white rhinoceros,

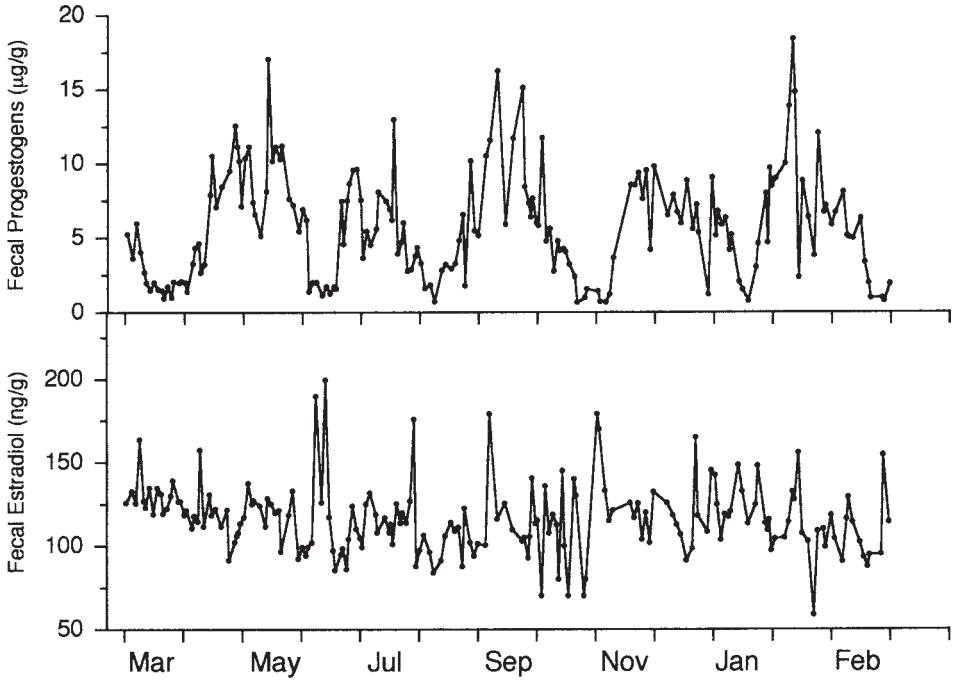

Fig. 8. Individual profiles of fecal progestogen and estradiol concentrations in a white rhinoceros female.

black rhinoceros fecal extracts did not contain measurable immunoactivity associated with fractions containing the androstenedione or DHT reference tracers. Only a small amount of immunoactivity was associated with the androstanediol reference tracer.

In contrast to females, there was no age difference ($P > 0.05$) between black and white rhinoceros males in this study population. There was no evidence of seasonality ($P > 0.05$) in testicular steroidogenic activity in either species (Fig. 10). Overall mean fecal androgen concentrations were higher ($P < 0.05$) in the black (27.6 ± 6.9 ng/g; range, 2–300 ng/g) than in the white (16.8 ± 3.1 ng/g; range, 2–200 ng/g) rhinoceros, although there was some overlap in concentrations of weekly means. There was no difference ($P > 0.05$) in average fecal androgen concentrations between proven and unproven males for the black (25.8 ± 7.2 ng/g versus 27.1 ± 7.9 ng/g) or white (17.6 ± 4.9 ng/g versus 15.8 ± 5.1 ng/g) rhinoceros, respectively.

Black and White Rhinoceros Adrenal Activity

Numerous peaks of corticoid immunoreactivity were identified in HPLC-purified eluates of fecal extracts in the black and white rhinoceros using the corticosterone RIA (Fig. 9c,d). One of the peaks was associated with the corticosterone reference tracer; however, other unidentified metabolites of greater and lesser polarity also cross-reacted in the assay. Although immunoactivity was observed near the elution peak for cortisol, the corticosterone antibody has less than a 1% cross-reactivity with that corticoid.

After ACTH injection, serum cortisol increased from baseline (~10 ng/mL) to

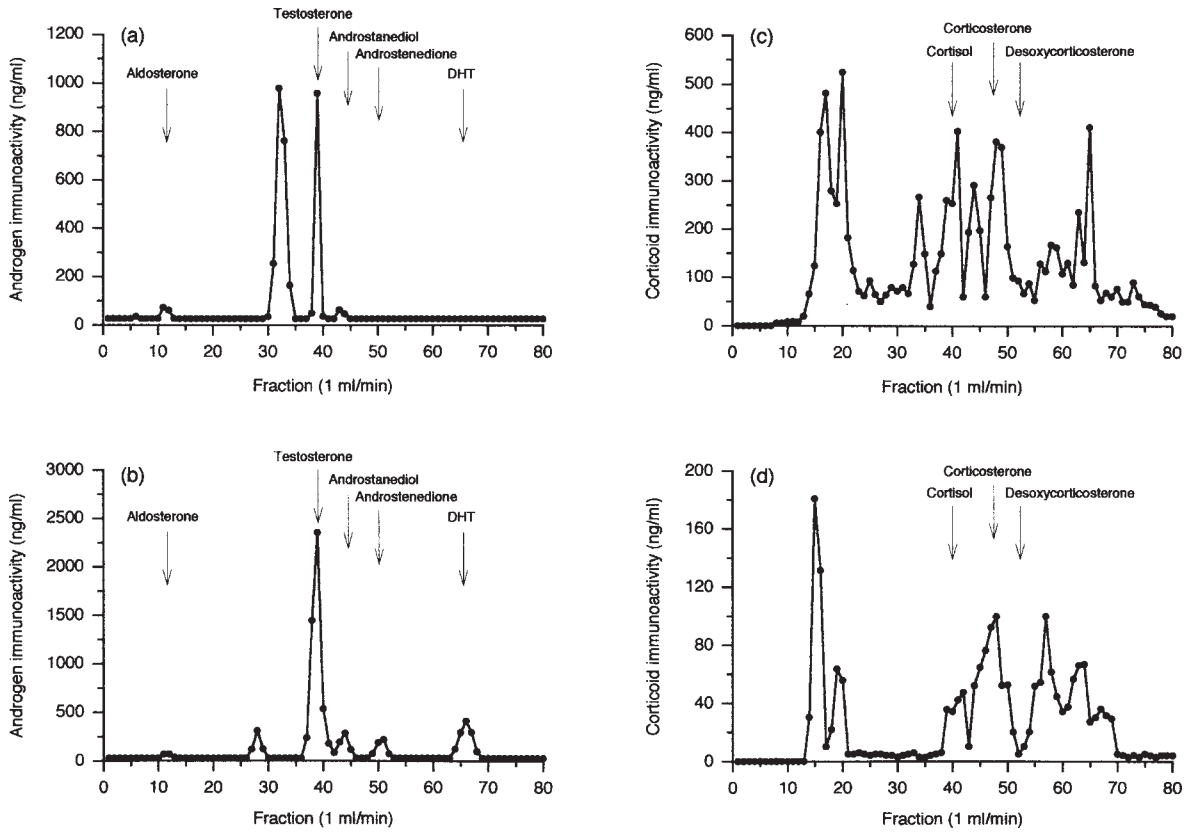

Fig. 9. High-pressure liquid chromatography analysis of fecal androgen (**a**, **b**) and corticoid (**c**, **d**) metabolites in the black (**a**, **c**) and white (**b**, **d**) rhinoceros, respectively. Retention times of immunoreactive peaks were compared to ^{3}H-steroid reference tracers.

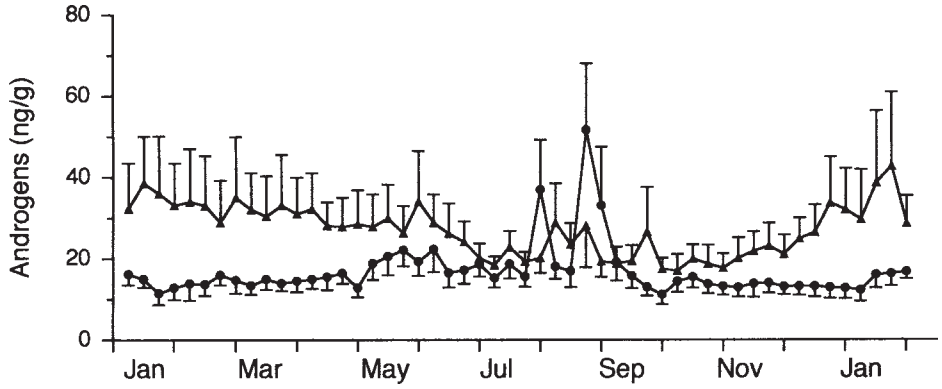

Fig. 10. Weekly mean (±SEM) profiles of fecal androgen concentrations in black (solid triangles; n = 10) and white (solid circles; n = 6) rhinoceros males.

103 and 161 ng/mL in the two black rhinoceroses subjected to blood collection. Using the DPC cortisol assay, no corticoid immunoactivity was detected in fecal dilutions, nor was a post-ACTH increase observed. Conversely, using the ICN corticosterone RIA, a several-fold increase in corticoid immunoactivity was measured in fecal extracts within 2 days after ACTH injection (Fig. 11).

On the basis of weekly fecal sample analysis, corticoid metabolite concentrations fluctuated within and among individuals, with no indication of seasonality in adrenal activity (Fig. 12). Within species, there were no gender differences ($P > 0.05$) in overall mean corticoid concentrations in black (male, 46.0 ± 3.5 ng/g; female, 39.2 ± 4.9 ng/g) or white (male, 32.0 ± 3.1 ng/g; female, 30.9 ± 1.2 ng/g) rhinoceroses. However, overall mean concentrations were higher ($P < 0.05$) in the black (41.8 ± 3.0 ng/g) compared to the white (31.2 ± 1.7 ng/g) rhinoceros, with this difference being more prominent in the males (Fig. 12a).

There were no correlations ($P > 0.05$) between fecal corticoids and concentrations of progestogens in females or androgens in males ($r = 0.12$ and 0.09, respectively). Furthermore, in the black rhinoceros there was no difference ($P > 0.05$) in overall mean fecal corticoid concentrations between times females were cycling (45.7 ± 2.1 ng/g) or not (42.9 ± 4.3 ng/g). There also was no difference ($P > 0.05$) in fecal corticoids in the white rhinoceros between females exhibiting no ovarian activity (29.3 ± 5.7 ng/g) and those that exhibited at least some cyclicity (29.1 ± 2.4 ng/g).

DISCUSSION

This study is the first to directly compare longitudinal profiles of excreted gonadal and adrenal steroids in both species of African rhinoceros. Large numbers of animals were evaluated across multiple institutions to examine the reproductive status of the North America ex situ populations. As anticipated, on the basis of present breeding success rates, the white rhinoceros appears to be reproductively compromised and demonstrates abnormal ovarian cyclicity. Additional new data were generated on the lack of a definitive measurement to assess follicular activity, the similarity in androgenic patterns of proven versus unproven males, and species-specific differ-

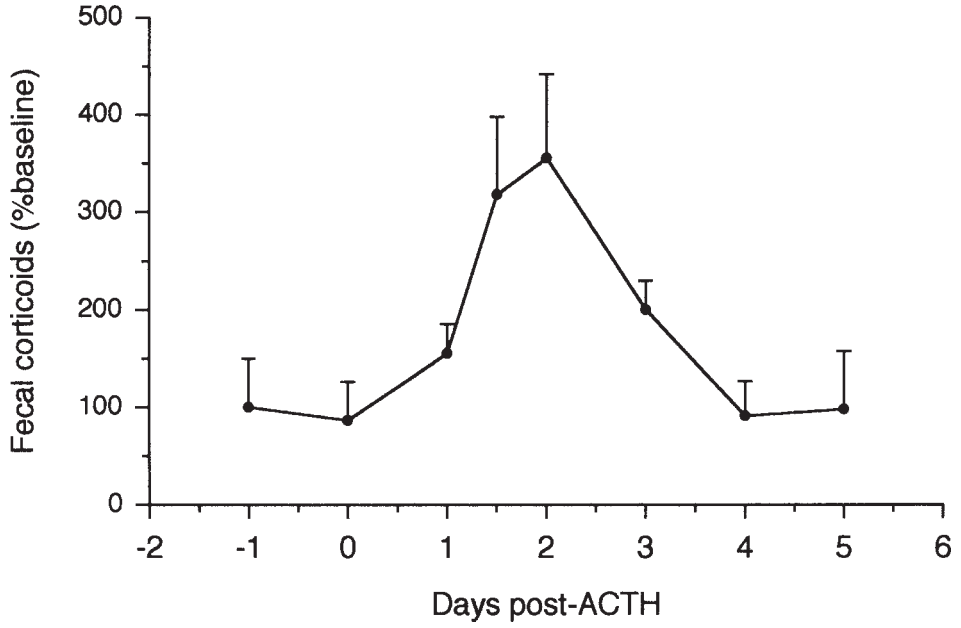

Fig. 11. Mean (±SEM) fecal corticoid response to ACTH injection (800 IU, gel formulation) in four black rhinoceros males. Data are presented as a percent of baseline values and aligned to the time of injection.

ences in corticoid levels. All of this information emphasizes the challenges in interpreting and using hormonal data to enhance the management of these species.

For the black rhinoceros, Hindle et al. [1992] reported a 21–22-day estrous cycle on the basis of urinary 20α-dihydroprogesterone analysis. However, subsequent fecal progestogen monitoring supports the present finding of a 26-day cycle [Schwarzenberger et al., 1993, 1996; Berkeley et al., 1997; Radcliffe et al., 2001]. Past studies have largely focused on a few animals in which estrous cyclicity was monitored for relatively short periods (generally <6 months). No hormonal data were available to determine whether black rhinoceros females in North America cycled regularly or were affected by season. Present results suggest that most females do cycle throughout the year. However, estrous cycle lengths varied, with approximately 39% falling outside a normal range (20–32 days). Unexplained, sporadic periods of ovarian quiescence also were observed, but were transient and apparently had a minimal effect on fertility. In a study of ~70% of the black rhinos in North America, Carlstead et al. [1999] identified three predictors of reproductive success: 1) females being behaviorally dominant to the male; 2) large enclosure area (>1,000 m^2); and 3) a minimal amount of high concrete wall exhibit construction. Integrating all available information, the data suggest that suboptimal reproductive success in the black rhinoceros is unrelated to a consistent lack of ovarian activity. On the contrary, social or environmental cues are likely factors reducing fecundity in the ex situ black rhinoceros population. It also is recognized that high calf mortality in captive black rhinoceroses remains a considerable problem [Foose and Reece, 1997; Foose, 1999].

During gestation in the black rhinoceros, fecal progestogens were at luteal phase concentrations for the first several months, then increased markedly similar to previ-

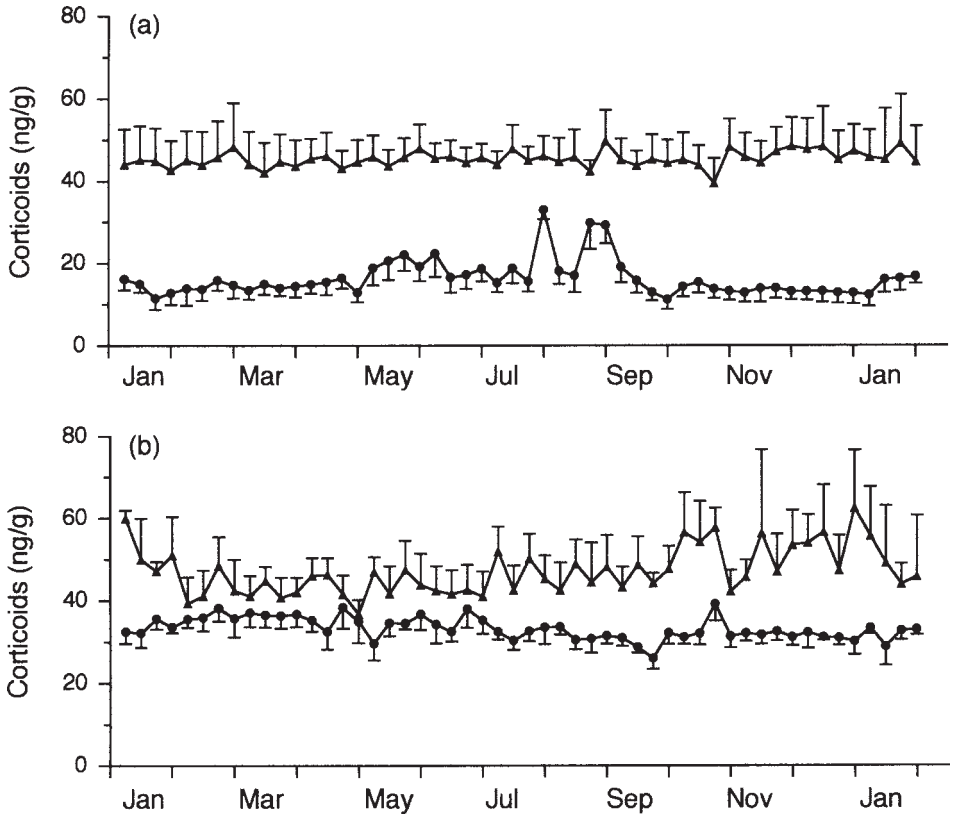

Fig. 12. Weekly mean (±SEM) profiles of fecal corticoid concentrations in male (**a**) and female (**b**) black (solid triangles; n = 10) and white (solid circles; n = 6) rhinoceros.

ous reports [Schwarzenberger et al., 1993, 1996; Berkeley et al., 1997; Garniera et al., 1998; Radcliffe et al., 2001]. Fecal progestogens declined to baseline 2–3 days after birth, reflecting a lag in steroid excretion because of gastrointestinal transit time [Schwarzenberger et al., 1993; Radcliffe et al., 2001]. In three females, progestogen concentrations remained low for the first 3–5 months of lactation, after which cyclicity resumed despite continued nursing by the calf until approximately 1 year of age. A similar postpartum anestrus has been reported by Schwarzenberger et al. [1993]. However, we observed two occasions in which an increase in progestogen excretion occurred approximately 1 month after parturition, suggesting a postpartum ovulation. Other Perrisodactyla (e.g., horses and tapirs) recycle shortly after parturition and can conceive, although this cycle generally is considered less fertile [Ginther, 1992; Brown et al., 1994a]. The postpartum ovulation observed in these black rhinoceros females did not always result in resumed cyclicity, however, because two returned to an anestrus state for at least the next 6 months. One female gave birth at the beginning of the study and conceived again 1 year later, resulting in an interbirth interval similar to the 27 months reported for wild rhinoceroses [Joubert and Eloff, 1971], but shorter than the 40-month average reported for other captive females [Smith and Read, 1992].

In contrast to the black rhinoceros, few white rhinoceroses in captivity are proven breeders, whether males or females [Foose and Reece, 1997; Foose, 1999]. This

species has been the target of several endocrine-monitoring studies, so that currently more "profiles" are available for the white than for the black rhinoceros. Despite this effort, defining the normal estrous cycle for this species remains problematic. Estrous cycle lengths have been estimated to be 30–90 days when based on behavioral observations [Owen-Smith, 1975], 25–42 days using urinary steroid analyses [Hindle et al., 1992], and 26–70 days through fecal progestogen evaluations [Radcliffe et al., 1997; Schwarzenberger et al., 1998; Patton et al., 1999]. The latter reports plus the present data confirm that the white rhinoceros exhibits two estrous cycle types, one ~30 days and the other ~70 days in length [Radcliffe et al., 1997; Schwarzenberger et al., 1998; Patton et al., 1999].

An ensuing debate over which cycle type is "normal" now is under way. Using ultrasonography and fecal progestogen analyses, Radcliffe et al. [1997] observed two nonconceptive cycles of 31 and 35 days, and two long cycles of 73 and 78 days in a single white rhinoceros female. The long cycles were confirmed to be the result of embryonic loss and so were designated abnormal because of specific pathological factors. Schwarzenberger et al. [1998] evaluated 21 females that were classified into four categories: 1) regular 10-week estrous cycles (n = 2); 2) variable cycles of 4 or 10 weeks (n = 6); 3) no cycle regularity, but some evidence of luteal steroidogenic activity (n = 6); and 4) no luteal activity (n = 7). Although the majority of females exhibited erratic or no luteal activity, most of the "regular" cycles lasted 10 weeks; thus, the long cycle was designated normal. A third study of 13 white rhinoceros females confirmed the presence of long and short cycles [Patton et al., 1999]. Again, most of the females exhibited erratic or no luteal activity (8 of 13), but five females exhibited cycles that were categorized as type I (~30-day duration; n = 10) or type II (~10 weeks; n = 7). Two females exhibited both cycle types, similar to the category 2 animals described by Schwarzenberger et al. [1998], but most of the cycles were short. Because the reproductive rate of white rhinoceroses at this facility (San Diego Wild Animal Park) was comparatively high, it was concluded that the short cycle pattern likely was normal [Patton et al., 1999].

Of the cycles observed in our study, the majority were long (83%) and the data more comparable to those of Schwarzenberger et al. [1998]. However, this similarity does not mean that only the long cycle is normal. In fact, conception has been documented in females exhibiting both short and long cycles [Schwarzenberger et al., 1998] and in those with short cycles only [Radcliffe et al., 1997; Patton et al., 1999]. No females conceived in our study, but mating was observed before long and short luteal phases. In defense of the short cycle as normal, Patton et al. [1999] suggested that extended luteal phases could be the result of pyometra, endometritis, or embryonic loss, all of which have been diagnosed in white rhinoceroses exhibiting prolonged progestogen excretion [Radcliffe et al., 1997; Patton et al., 1999]. In defense of the long cycle as normal, Schwarzenberger et al. [1998] referred to data of Radcliffe et al. [1997] to propose that some short luteal phases associated with low progestogen production were caused by cystic or hemorrhagic follicles and anovulation. In a recent transrectal ultrasound evaluation of 14 white rhinoceros females (4–30 years of age), Hermes et al. [2000] identified a number of reproductive abnormalities designated as "types": type I, small inactive ovaries accompanied by a fluid-filled uterus; type II, persistent luteal structures; type III, ovaries containing follicular cysts; and type IV, subadult females that exhibited follicular development but no ovulation. Assuming that abnormal cycles have a physiological cause, it is surprising that one or the other is not

more irregular in duration. In horses, extended luteal phases from diestrous ovulation or uterine abnormalities often result in highly variable cycle lengths [Ginther, 1992], and cystic follicular structures, ovulatory or not, do not always regress consistently. Likewise, early embryonic death is not a precisely timed event [Ginther, 1992]. To add to the confusion, data from a female Sumatran rhinoceros (*Dicerorhinus sumatrensis*) suggest that this species may be an induced ovulator [Roth et al., 2001]. Ultrasound examinations revealed the formation of anovulatory hemorrhagic follicles that grew beyond preovulatory size in the absence of mating. These follicles underwent varying degrees of luteinization that resulted in irregular fecal progestogen profiles; yet, when allowed to mate, the female showed regular 21-day cycles. In our study, because both cycle types were observed after natural mating, the degree of ovulatory stimulus would not appear to be a factor in determining cycle normality.

Whatever the cause of variable cyclicity in the white rhinoceros, it is clear that these reproductive problems need to be addressed. A high priority must be to use noninvasive hormone monitoring in conjunction with frequent ovarian examinations via ultrasound to document the physiology (or pathology) associated with each cycle type. More demographic information also is needed to determine whether age is a factor. In horses, endometritis and early embryonic death increase with animal age [Carnevale and Ginther, 1992]. Based on ultrasonography, only four females in the study of Hermes et al. [2000] were free of reproductive abnormalities and they were all less than 11 years of age. In our study, only two females were less than 25 years of age. One female (11 years old) displayed a sporadic progestogen profile and no clear cyclicity, whereas the other (20 years old) exhibited two short successive cycles, but otherwise was anovulatory. Conversely, the two females that cycled year round were both in their upper 20s. Obviously, age alone cannot not account for all of the reproductive problems observed in captivity. Still, considering the already advanced age of the adult white rhinoceros population in North America, trouble with erratic or totally quiescent ovarian activity likely will continue to worsen.

Evaluating fecal estrogens provided no useful assessment of the follicular phase for either species. Berkeley et al. [1997] reported that fecal estrogens in one black rhinoceros correlated with serum estradiol, and a fecal estradiol peak occurred coincident with breeding. However, there were no significant correlations between specific estrous behaviors and fecal estrogen profiles. In our study, occasional estrogen peaks were observed preceding increased fecal progestogens and in some cases breeding, but the patterns were inconsistent even when daily samples were collected. Furthermore, random estrogen surges occurring at other stages of the cycle were not uncommon. The lack of an increase in estrogen production during gestation [Berkeley et al., 1997; this study] suggests that these rhinoceros species may differ from other Perissodactyla (e.g., tapirs, horses), where this measurement is a useful pregnancy diagnosis tool [Bamberg et al., 1991; Schwarzenberger et al., 1991; Chapeau et al., 1993]. In this study, we used a total-estrogens assay for the black rhinoceros and an estradiol assay for the white rhinoceros in an effort to select the most appropriate system for each species. The choices were based in part on the data of Berkeley et al. [1997] where "total estrogens" were measured in the black rhinoceros, and on findings in the white rhinoceros where estradiol-17β was a major excretory product in feces [Hindle and Hodges, 1990]. Although it is possible that an appropriate assay system reflecting ovarian/placental estrogen production has yet to be identified, it is just as conceivable that

fecal estrogen analysis will never be an effective means to identify estrus or diagnose pregnancy in these species. Rather, urinary estrogen monitoring appears to be more practical for these purposes as suggested by Hindle et al. [1992], who reported that distinct peaks coincided with behavioral estrus when hydrolyzed urine was assayed for estrone (black rhinoceros) or estradiol-17β (white rhinoceros).

Reproductive cycles were observed during every month of the year in the black and white rhinoceros. Other endocrine studies [Hindle et al., 1992; Schwarzenberger et al., 1993, 1996, 1998; Berkeley et al., 1997; Patton et al., 1999] and captive breeding records [Foose, 1997; Foose and Reese, 1997; Foose, 1999] also support a lack of seasonality in these species, although birth peaks have been noted [black: October–January, March, August; white: April–July, November–January; Fouraker and Wagener, 1996]. Fecal androgen analyses further revealed no evidence that these rhinoceroses were seasonal. Advanced aged males (>25 years old) also produced androgens at concentrations comparable to younger counterparts, which supports studbook records indicating that animals can remain fertile until at least that age [Fouraker and Wagener, 1996]. There was a significant difference in overall mean fecal androgen concentrations between the black and white rhinoceros that may be related to differences in the androgen immunoactivity profile of HPLC-purified extracts. Both species excreted a metabolite that eluted at the position of testosterone, but there was an equally large unidentified immunoactive peak in the black rhinoceros that was barely detectable in the white rhinoceros. Conversely, immunoactivity, albeit minor, occurring around the elution time of DHT was observed only in the white rhinoceros.

Creating self-sustaining zoo populations of rhinoceroses requires understanding the causes of irregular cyclicity and developing strategies to stimulate breeding. Achieving appropriate social groupings and stimulating reproductive behavior likely are essential. In the white rhinoceros, nonbreeding females exposed to new males within the same facility or translocated to a new facility have become breeders [Patton et al., 1999]. The most compatible pairs of black rhinoceros are those in which the female is dominant to the male [Carlstead et al., 1999]. Another potential factor is "stress" as an underlying cause of poor reproductive performance in captivity [Foose and Reece, 1997]. The ultimate impact of a stressor depends on the subjective perception of the threat [Hennesy and Levine, 1979; Levine, 1985; Mason, 1968] modulated by the complexity of its environment and the response options it has [Levine, 1983; Levine et al., 1979]. Although a variety of potential stressors exist in zoos, the deleterious effects occur only if an animal is unable to cope (e.g., hide, flee, attack, etc.) [Weiss, 1968, 1971]. Determining whether environmental conditions facilitate or compromise coping is key to ensuring animal well-being. Thus, we have taken the first step in identifying an assay system capable of assessing adrenal activity as a potential indicator of stress in the black and white rhinoceros. Our data indicated that immunoactive corticoids were measurable in rhinoceros feces by using the same assay system that has proven reliable for monitoring adrenal function in a diverse array of species [see review, Wasser et al., 2000]. This approach (i.e., measurement of fecal glucocorticoids) is increasingly being used to investigate the effects of various stressors on animal physiology [see review, Whitten et al., 1998], and will be key to future studies of rhinoceros reproduction.

This initial study indicated that fecal corticoid concentrations are ~30% higher in the black than in the white rhinoceros. Whether this is related to species or age differences in adrenal function or stress responsiveness remains to be determined. No differ-

ences in adrenal activity were noted in black or white rhinoceros females when related to cyclicity status, and there were no significant correlations between fecal corticoids and overall concentrations of gonadal steroids in males or females. However, these results do not mean that changes in adrenal activity have no impact on reproductive function in the rhinoceros. We conclude that because fecal corticoid concentrations fluctuate over time, an adequate sampling regimen must be used for accurate assessments of baseline adrenal activity (e.g., at least weekly for yearlong evaluation trials; more frequently for shorter studies). Conducted properly, these assessments likely will be most useful when data are compared with previously established baseline values to evaluate individual responses to changes in management strategies or environmental factors.

In conclusion, a new field of investigation is emerging in zoo biology that aims to raise the living standards of animals in zoos. One of the major focuses of zoo animal welfare science will be the measurement of well-being based on a combination of assessment criteria related to biological functioning, natural behavior, and subjective emotional states. For rhinos in captivity, where space is restricted, problems have been identified that suggest compromised animal well-being (e.g., poor reproductive performance, disease susceptibility, and a mortality rate that exceeds birth rate). These problems are believed, in part, to be related to the animals' inability to cope with adverse environmental and social conditions, and for this reason assessing "stress" in captive rhinos is a high priority [Foose and Reece, 1997]. Thus, future studies will involve integrating endocrine assessments, such as fecal corticoid analyses, with other evaluations based on behavior, reproductive physiology, immunology, and pathology to potentially provide a more meaningful measure of stress and associative factors affecting health and reproduction.

CONCLUSIONS

1. Most black rhinoceros females were cycling, whereas many white rhinoceros females exhibited periods of acyclicity unrelated to season.

2. Cycling white rhinoceros females predominantly exhibited long estrous cycles of approximately 70 days. Determining whether the "long" or "short" duration estrous cycle represents abnormal reproductive function will require concomitant endocrine and reproductive tract ultrasound analyses of reproductive-age females.

3. Monitoring fecal estrogen excretory patterns was not useful for characterizing the follicular phase or estrus in either rhinoceros species.

4. Testicular activity, assessed by fecal androgen analysis, was not affected by season, age, or breeding status, but did differ between species, with concentrations being higher in the black than in the white rhinoceros.

5. Fecal corticoid concentrations were slightly but significantly higher in the black than in the white rhinoceros.

6. Because the age demographics of the white rhinoceros population in this study was skewed toward older individuals, it remains to be determined what effect age has on the comparative differences observed in steroid profiles between species.

ACKNOWLEDGMENTS

We thank the keepers and veterinary staff of all participating facilities for assistance with behavioral observations and sample collection (Cameron Park

Zoo, Caldwell Zoo, Cincinnati Zoo, Dallas Zoo, El Coyote, Fort Worth Zoo, Fossil Rim Wildlife Center, Knoxville Zoo, Milwaukee Zoo, North Carolina Zoo, Riverbanks Zoo, San Antonio Zoo, White Oak Conservation Center, and The Wilds). We are especially grateful to Wieke Galama, Sue Walker, and Tanya Moeller for excellent technical assistance in sample processing and endocrine analyses. A special thanks also is extended to Jeff Stehle at the San Antonio Zoo for testing the fecal markers. This project was funded by a generous grant from the International Rhino Foundation.

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