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Serum and Fecal Steroid Analysis of Ovulation, Pregnancy, and Parturition in the Black Rhinoceros (*Diceros bicornis*)

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Studies were conducted to determine: 1) if fecal hormone metabolite concentrations correlated with serum estrogen and progesterone concentrations, follicular activity and reproductive behavior in the black rhinoceros (*Diceros bicornis*) and 2) if threshold values of respective fecal metabolite concentrations correlated with pregnancy. Blood and fecal samples were collected, in conjunction with transrectal ultrasound and behavior observations, for an 18-month period from one black rhinoceros female. Subsequently, serial fecal samples were collected from 13 females in 10 zoos. Quantitative analysis of serum progesterone (P_4) and estradiol (E_2) was performed by radioimmunoassay (RIA); analysis of fecal estrogen metabolites (E) and fecal progesterone metabolites (P) were performed by enzyme immunoassay (EIA). Serum P_4 concentrations identified two luteal phase patterns and two nadirs which corresponded with behavioral estrus. Fecal E patterns indicated a sharp peak which corresponded with breeding. Concentrations of fecal P illustrated identifiable nadirs and several peaks which corresponded to serum P_4 nadirs and luteal phases. Serum P_4 concentrations were not different between the luteal phase and pregnancy. Fecal P concentrations started to rise above luteal phase concentrations approximately 150 days postbreeding and remained elevated until immediately before parturition. Serum E_2 and fecal E concentrations rose and subsequently declined after parturition. In the fecal samples from seven pregnant females, fecal P concentrations were similarly elevated compared to six nonpregnant females. Results indicated that fecal steroid metabolites accurately reflected serum steroid hormone concentrations and that the measurement of P and E concentrations permitted the characterization of the estrous cycle, the diagnosis of pregnancy, and the onset of parturition. Zoo Biol 16:121–132, 1997. © 1997 Wiley-Liss, Inc.

Key words: reproduction; Perissodactyla; hormones; progesterone metabolites

INTRODUCTION

Maintaining viable populations of endangered species is important to zoological institutions. While knowledge of population dynamics is necessary for wildlife management, detailed assessments of reproductive function are required for long-term propagation of a captive species [Lasley et al., 1994]. One species in need of additional knowledge about reproductive endocrine patterns is the black rhinoceros (*Diceros bicornis*). Reproductive behaviors have been studied [Goddard, 1966; Hitchins and Anderson, 1983], but the intractable nature of this species had made acquisition of information about reproductive physiology difficult. Behavioral observations have provided conflicting data about age of sexual maturity and interestrous intervals [Hitchins and Anderson, 1983], necessitating the need for correlative physiologic data. Noninvasive methods, such as measurement of fecal steroid concentrations, can provide information regarding onset of sexual maturity, occurrence of ovulation and pregnancy, fetal loss rates, onset of parturition, and even occurrence of neonatal loss [Kirkpatrick and Lasley, 1991].

Steroid analysis of excreta has been applied to different species of rhinoceroses [Hodges and Green, 1989], including the black [Czekala, 1996; Ramsay et al., 1987, 1994; Hindle et al., 1990, 1992; Schwarzenberger et al., 1993, 1996], white (*Ceratotherium simum*) [Hodges and Hindle, 1988; Hindle and Hodges, 1990; Schwarzenberger et al., 1994; Walzer and Schwarzenberger, 1995] and Indian (*Rhinoceros unicornis*) [Kassam and Lasley, 1981; Kasman et al., 1986]. In none of these studies, however, were fecal or urinary endocrine patterns validated with serum hormone values or ultrasound characterization of ovarian events. Therefore, the hypotheses in this study were that (1) fecal reproductive steroids or their metabolites reflect serum reproductive steroids during the estrous cycle and pregnancy, (2) fecal steroids or their metabolites which reflect estrus coincide with breeding behaviors, (3) changes in serum estradiol and progesterone during the estrous cycle and pregnancy are consistent with ultrasound evaluation of ovarian follicles and fetal development, and (4) fecal steroid analysis using the P EIA accurately diagnoses pregnancy in the black rhinoceros.

METHODS

Study 1

Study animals. A 29 year-old multiparous black rhinoceros female at the Sedgwick County Zoo in Wichita, KS, was used for this study. She was housed alone in a pachyderm building with two adjoining indoor stalls that led to an outside yard. A restraining chute for blood collection and use of ultrasound consisted of collapsible aluminum gates set in the transfer passages of the building. Fecal and serum samples were collected from March 31, 1992 (day −64) to September 1, 1993 (day 486), with day 0 = day of mating. The female had calved successfully twice (her last calf was born in 1976), but the last 3 pregnancies ended in spontaneous abortions (at 9 months of gestation in October 1989, at 10 months in April 1991, and at 3 months in December 1991). The reasons for these abortions were unknown, but may have included poor body condition or luteal/placental progesterone insufficiency. Given this background, once pregnancy was confirmed by transrectal ultrasound, certain precautions were taken.

These precautions included: 1) mulching her daily diet of hay in order to make it easier for her to chew, and 2) 20 mls of a synthetic progesterone, Regumate (2.2 mg altrenogest/ml oil suspension, Hoechst-Russel, Inc.) was added to her daily diet after pregnancy was confirmed until before parturition (days 144 to 454 of pregnancy). The male was 9 years old at the onset of the study. He was the sire of the last three pregnancies that aborted. Initial attempts to introduce the male to the female in the outside enclosure were unsuccessful due to aggression by the female until eleven days before breeding occurred.

Serum collection and analysis. Blood samples were collected via venipuncture in alternating forelegs approximately twice per week. The serum was recovered and stored at $-20°C$ until analysis. Quantitative analysis of serum estrogens (E_1) and progesterone (P_4) were performed using commercial radioimmunoassay kits (Coat-a-Count, Diagnostic Products Corporation, Los Angeles, CA), according to the kit instructions. While the primary estrogen cross-reacting with the estrogen antibody was 17β-estradiol, the results are assumed to represent total immunoreactive estrogens. Similarly for the progesterone RIA, the results are reported as P_4, but may include other immunoreactive progestagens as well. To validate the repeatability of these assay kits for use in the rhinoceros, serially diluted (1:1, 1:2, etc.) serum samples (n = 4) were assayed and compared to the standard curve for evidence of parallelism.

Ultrasonography. Transrectal ultrasound for monitoring ovarian activity was performed using an Aloka 500V (Corometrics, Wallingford, CT) with a 5 MHz linear-array or 3.5 convex transducer [Schaffer and Beehler, 1990]. Ultrasound examinations were performed at three days before breeding and at 13, 55, and 121 days postbreeding.

Behavioral observations. Behavioral data were collected for a minimum of one hour/day from March to July 1992 (days −63 to 48 of sample collection) for a total of 121 hours. Fourteen behaviors were measured, using a "one-zero" sampling method at 15 second intervals (Table 1). All behaviors were tabulated as frequency of observed behavior per total number of observation intervals [Bernstein, 1991]. Regression analysis and Pearson's correlation coefficient were used to determine if quantitative changes in observed behavior frequencies were related to changes in serum and/or fecal hormone concentrations.

TABLE 1. Definitions of behaviors used in Study 1

Urine spraying	of short duration, bursts of urine spray, not eliminative urination.
Flehmen	head raised, underside of upper lip curled up, usually occurs after urine investigation
Rubbing against object	rubbing against object with horn
Following	travels behind, within 1.5 body lengths of other animals, or parallel, within 1 body length of other animal
Anogenital investigation	sniffs anogenital region of another animal
Vulvar winking	rapid contractions of vulva, often associated with urine spraying
Foot scraping	rapid shuffle of hind legs on ground, while animal remains stationary, often associated with defecation
Head resting	male resting head on female's back or hindquarters
Mounting	male weight on hind legs, head over female's back or hindquarters
Jousting	lateral movement of head contacting other rhino on head or horn
Hind-leg dragging	animal walks with hind legs stiff and straight behind
Pacing	repetitive locomotion within a specific area
Urine investigation	focal animal sniffing urine of another animal
Head sweeping	head is to the ground, moved laterally, while rooting air with horn

Fecal collection and sample preparation. Fresh fecal samples (5 g) were collected daily, placed into a plastic bag and frozen until analysis. After thawing, 0.5 g was weighed and placed in a pre-weighed scintillation vial containing 4 ml of aqueous extraction buffer [20% methanol, Shideler et al., 1993]. Samples were then placed on a shaker for 18 hr at room temperature. The resulting thick liquid was decanted into a test tube and centrifuged. After centrifugation, approximately 1 ml of the supernatant was decanted into prelabeled microcentrifuge vials and stored frozen until use. The pellet was returned to the scintillation vial and the contents of the scintillation vial were dried under air and weighed in order to determine the dry weight of the feces. The supernatant was loaded directly on the EIAs. If the results were above the highest standard, the extracts were diluted with assay buffer and re-assayed. All hormone concentrations are expressed as nanograms per gram dry feces (ng/g df). Estimated hormone recovery from the fecal samples was determined by adding known quantities of ^{3}H-estradiol (n = 5) and ^{3}H-progesterone (n = 5) to the wet fecal samples and extracting the samples as described above. The amount of radiolabeled hormone recovered from these samples was compared to an extracted sample with radiolabeled hormone added after the extraction procedure (n = 1 for each hormone).

Enzyme immunoassays. Two enzyme immunoassays (EIAs) were used: estrogen metabolites (E) and progesterone metabolites (P). The lowest standard for each assay was considered to be the limit of sensitivity for that assay (6.25 pg/well for the E assay and 15.6 pg/well for the P assay. All sample results that were below that value were recorded as zero. Estrogen metabolite immunoreactivity (Ab E1C 583) in the fecal extracts was measured using an assay described by Stabenfeldt et al. [1991]. Briefly, the immunogen was estrone-3-glucuronide bovine serum albumin, the major cross-reactivities include estrone sulphate (100%), estrone glucuronide (70%), estrone (269%), and estradiol-17β (9.8%). The intra- and inter-assay coefficients of variation were 10.2% (n = 8) and 11.1% (n = 9), respectively. Fecal extracts were assayed for P (Ab Po 4861) using an assay described by Munro and Stabenfeldt [1984]. Briefly, the immunogen was a progesterone 11α-hemisuccinyl-bovine serum albumin, the major cross-reactivities include: 11α-hydroxyprogesterone (21.4%), 5α-pregnan-3,20 dione (29.5%), and 20β-hydroxyprogesterone (2.38%). The intra- and inter-assay coefficients of variation were 16.4% (n = 8) and 8.9% (n = 6), respectively. Parallelism to the standard curve was performed for fecal E and P and serum E_2 and P_4 by assaying successive halving dilutions of selected fecal and serum samples (n = 4 samples for each test). These values were graphed against the standard curve of each assay to determine parallelism. Regression analysis and Pearson's correlation coefficient were used to determine if a lag relationship existed between the circulating serum estradiol and progesterone concentrations and their respective concentrations in the feces (Statview II, Abacus Concepts, Inc., Berkeley, CA 94704). The days of feces collection were normalized to day of serum collection (n = 47). The days examined were: 0 (day of serum collection), +1, +2, +3, +4, +5, +6. Mean values for hormone concentrations are reported ± standard error of the mean.

Study 2

Based on the results of Study 1, accuracy of fecal steroid analysis for the detection of pregnancy was tested using 13 sexually mature females housed at 10 zoos. Beginning at least 150 days after the last observed mating, 5 daily fecal samples were collected from each female over the course of a week. The stage at which

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sampling occurred during pregnancy was determined retrospectively from the time of parturition for each female, except for the one that aborted. The samples were analyzed for fecal P concentrations as described above. A Mann-Whitney U test was performed to determine statistical differences between pregnant and non-pregnant hormone concentrations.

RESULTS

Study 1

The extraction recovery efficiencies for fecal E and fecal P were 59.2% and 35.8%, respectively. The measurement of E_2 and P_4 in serially diluted serum samples and of E and P in fecal extracts all showed parallelism to the respective standard curves. Correlations between serum E_2 and fecal E were the highest by days +2 and +3 after measurement of serum E_2 (day −2: R = 0.569, p = 0.0001; day +3: R = 0.737, p = 0.0001, d.f. = 45), and declined thereafter. Correlations between serum P_4 and fecal P peaked on day +4 (R = 0.576, p = 0.0001, d.f. = 45) following measurement of serum P_4.

Although mounting and subsequent breeding occurred near the time of peaks in serum E_2 and fecal E concentrations, no significant statistical correlations were found between the observed behaviors and the hormone profiles during the three-month observation period (Table 1). Analysis of serum E_2 revealed two ovulations, with E_2 peaks followed by extended elevations of circulating P_4 (Fig. 1). The length of the complete estrous cycle, from E_2 peak to E_2 peak, was 26 days. The first E_2 peak (day −23) occurred whenthe female was separated from the male, and without observed changes in the female's behavior. The male was given access to the female's enclosure on day −16. However, due to aggression by the female, he did not go into the enclosure until day −11, when a slight increase in serum E_2 (20 pg/ml) was detected. Ultrasonographic evidence supported endocrine and behavioral data for the occurrence of ovulation and pregnancy. Ultrasonography of the ovary revealed an image of a large (30 mm) diameter follicle on the left ovary and an 18 mm corpus luteum on the right ovary on day
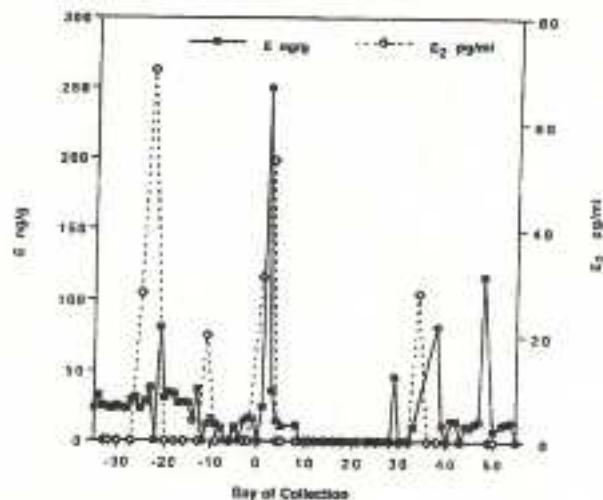

Fig. 1. Serum estradiol (E_2) and fecal estrogen metabolite (E) concentrations in a black rhinoceros female during the estrous cycle and early pregnancy (day 0 = day of mating).

−3, three days before breeding (day 0). Serum E_2 peaked on days 1 and 3, followed by a luteal phase P_4 elevation. Following this presumed ovulation, there was a rise in fecal P, signaling a luteal phase (Fig. 2). This was confirmed by ultrasound when, on day 10, a 22×35 mm corpus luteum was observed on the left ovary. On days 32 and 33, the female allowed the male to stand in close proximity to her and perform the head resting behavior, but not mounting was observed. A serum E_2 elevation was detected on day 34, followed by a rapid rise in fecal P concentrations. This elevation in fecal P, by day 133, was substantially greater than concentrations observed during the presumed luteal phases, and signaled the earliest point that pregnancy could be detected by distinguishing fecal P values from luteal phase P values (Fig. 3). The fetus was undetected by ultrasonography at either days 55 or 121 of pregnancy, although the embryonic sac, fetal membranes, and umbilicus were observed. Approximately two weeks prior to parturition, fecal P concentrations decreased to at luteal phase levels, or below. A live calf was born 468 days after mating.

Throughout the collection period both serum E_2 and fecal E concentrations were low. Fecal E was less than 75 ng/g df with five exceptions. The second peak on day 2 (250 ng/g df) occurred two days after copulation, and corresponded with an increase in serum E_2 concentrations (Fig. 1). A third increase occurred concurrent with a peak of serum E_2 (day 34), and this increase coincided with behavioral changes in the female, including increased acceptance of the male, standing for the male, and head-resting. The fourth period occurred during days 407–414 of sample collection, or about two months prior to parturition (Fig. 4). The fifth period of elevated E excretion occurred on the day before parturition and peaked at 171.4 ng/g df on the day of parturition (day 468). The final E elevation occurred 12–14 days after parturition.

Study 2

Of the 13 females tested for pregnancy by means of fecal P concentrations, 6 produced calves (Table 2). One of the calves was stillborn, and a seventh female had an apparent abortion. The mean fecal P concentration for the six confirmed pregnan-
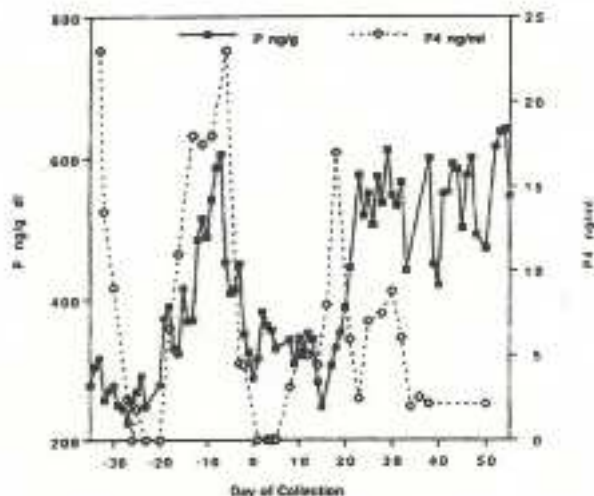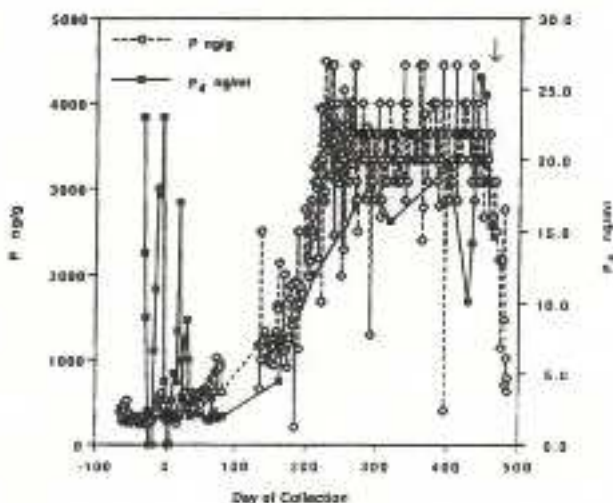

Fig. 2.   Serum progesterone (P_4) and fecal progestagen metabolite (P) concentrations in a black rhinoceros female during the estrous cycle and early pregnancy (day 0 = day of mating).

Fig. 3. Serum progesterone (P_4) and fecal progestagen metabolite (P) concentrations in a black rhinoceros female during the estrous cycle, pregnancy, and parturition (day 0 = day of mating, arrow indicates day of parturition).

cies was 2462.2 ± 453.6 ng/g df, which differed significantly ($U = 1$, $p = 0.004$) from the mean fecal P concentrations from nonpregnant animals (213.8 ± 93.5 ng/g df). More importantly, the lowest mean value for a pregnant animal (2865.2 ± 393.2 ng/g df) after approximately 8 months of gestation, was over four times greater than the highest mean value (568.4 ± 38.9 ng/g df) for a nonpregnant animal.

DISCUSSION

Study 1 shows that fecal E and P concentrations accurately reflected circulating serum concentrations and ovarian actiity of the black rhinoceros during the es-
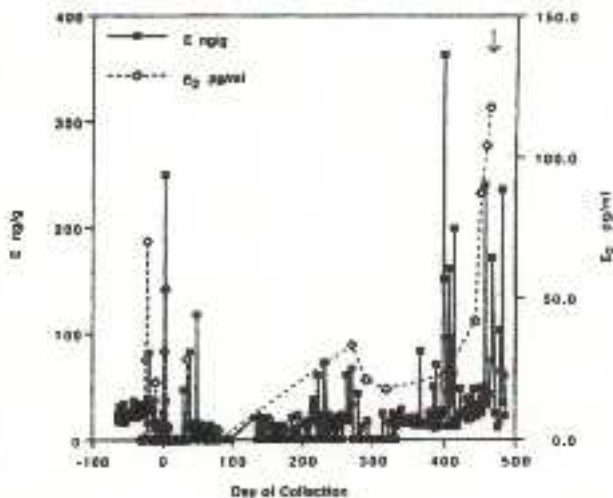

Fig. 4. Serum estrogen (E_2) and fecal estrogen metabolite (E) concentrations in a black rhinoceros female during the estrous cycle, pregnancy, and parturition (day 0 = day of mating, arrow indicates day of parturition).

TABLE 2. Fecal progestagen metabolite concentration (P) in black rhinoceroses producing (boldface) and not producing calves (normal type)

Studbook No.	P ng/g dry feces[a]	Approximate Days before Calving
400	**2865.2 (± 393.2)**	**196**
365	**2418.9 (± 75.9)**	**126**
53	**3504.1 (± 142.4)**	**186**
418	**2817.4 (±203.9)**	**30**
405	**2638.3 (± 427.3)**	**30**
351	**529.4 (± 14.8)**	**210**
364	**2711.6 (±122.9)**	**N/A[b]**
294	78.8 (± 3.7)	–
317	65.6 (± 6.2)	–
202	107.6 (± 8.5)	–
396	374.3 (± 65.5)	–
255	88.2 (± 8.4)	–
55	568.4 (± 38.9)	–

[a]Mean concentrations reported (± standard error)
[b]This female was diagnosed pregnant by transrectal ultrasound 140 days before fecal sample collection and subsequently aborted.

trous cycle and pregnancy. Serum E_2 and P_4 concentrations were markedly lower than in another published report [Kock et al., 1991] and may reflect antibody detection differences. The ultrasonography findings are consistent with other studies in rhinoceroses [Adams et al., 1991; Schaffer et al., 1990]. Further, fecal P concentrations signaled the existence of a pregnancy and distinguished pregnancy from luteal phase fecal P concentrations by day 150 of pregnancy.

The value of fecal P analysis for the detection of pregnancy is apparent by the different profiles in serum P_4 and fecal P concentrations over the course of both the estrous cycle and pregnancy in Study 1. Serum P_4 concentrations remained similar during the luteal phase and pregnancy, while fecal P concentrations during pregnancy exhibited a 4- to 10-fold increase over luteal phase levels. This increase in fecal P concentration is similar to that measured by Schwarzenberger et al. [1993], probably due to differences in pregnant vs. nonpregnant production and/or metabolism of steroid hormones. However, caution must be used when comparing the actual values because of differences in antibodies and assays. Other factors that may affect the results includes individual animal variation, genotypic differences, and different diet composition.

The administration of the synthetic progesterone Regumate to the female may have been a possible confounding factor in Study 1. This progestin may have been responsible for the elevated fecal P concentrations. However, three factors suggest that the Regumate did not significantly alter the fecal P pattern during pregnancy. First, fecal P concentrations continued to increase, despite a constant dose (about half the dosage recommended for horses) of Regumate during treatment. Second, fecal P concentrations for the Regumate treated female in Study 1 were within the same range as the nonRegumate treated pregnant females studied in Study 2. Third, it has been reported previously that the administration of a synthetic progestin did not affect fecal progestagen EIA measurements in a white rhinoceros [Walzer and Schwarzenberger, 1995].

The inability to accurately detect pregnancy by means of fecal E in the black rhinoceros is in contrast to the ability to use this same estrogen metabolite to detect pregnancy in other members of the Perissodactyla. This difference reflects the importance of phylogenetic variability with regard to the measurement of reproductive steroid metabolites. Fecal E has been used to successfully diagnose pregnancy in horses and tapirs [Bamberg et al., 1984, 1991; Chapeau et al., 1993; Kirkpatrick et al., 1991; Schwarzenberger et al., 1991]. The episodic E excretion during days 407–414 is unexplained and requires further investigation. The pre-partum E elevation is similar to other species such as the cow and the sheep [Catchpole, 1969]. Kock et al. [1991] reported that serum estradiol concentrations were higher in black rhinoceros females later in pregnancy than in early pregnant or nonpregnant animals.

Most interesting is the postpartum fecal E increase 12 to 14 days after parturition. This particular elevation suggests the maturation of a new group of follicles and resembles the "foal heat" seen in horses. Although Schwarzenberger et al. [1993] did not measure fecal estrogens, it was noted that fecal progestagens rose to luteal phase concentrations in two animals at 7–14 days and 22–36 days postpartum, respectively. Field observations of 40 female Namibian black rhinoceroses indicated that three had intercalf intervals of 16, 18, and 19 months [Joel Berger, pers. comm.] which are supportive of a possible postpartum estrus. The ability to detect a postpartum estrus may be important for artificial insemination attempts in order to decrease inter-birth intervals in captive management situations.

The inability to determine a statistically significant correlation between the behavioral data and the hormone concentrations is unfortunate but is most likely due to the sampling frequency. Captive conditions may be another factor: small enclosure size and/or masking of olfactory cues by frequent cleaning may not provide optimal conditions for normal behaviors.

The results of Study 2 confirm that it is possible to distinguish between pregnant and non-pregnant black females on the basis of fecal P concentrations, supporting the results of Schwarzenberger et al. [1993]. Based on keeper records and the limited sampling collection, it was not possible to distinguish between regularly cycling animals and animals with erratic or irregular cycling intervals. Irregular cycling intervals have been observed in other black rhinoceroses (Berkeley, 1994), and white rhinoceroses [Schwarzenberger et al., 1994]. It is apparent that serial samples need to be collected during early gestation to determine if fecal progesterone concentrations are actually increasing. Later pregnancy detection is possible with one or two fecal samples due to the great increase in excreted P in the feces.

In Study 2, EIA analysis of fecal P concentrations was 92% accurate (12/13) for predicting pregnancy approximately 150 days postconception. One pregnant female (studbook #351) had P concentrations in the nonpregnant range. While fecal steroid studies have not reported this phenomenon in the horse mare, transient low serum P_4 concentrations have been reported [Perkins et al., 1993; Schwab et al., 1990; Shideler et al., 1982]. In the study by Perkins [1993], wide diurnal variations of circulating P_4 concentrations were reported within individual mares, though consistently low circulating P_4 concentrations have been associated with an increased frequency of pregnancy loss [Schwab et al., 1990; Shideler et al., 1982]. To increase accuracy for pregnancy detection in the black rhinoceros, it is suggested that fecal samples be analyzed for P content at repeated monthly intervals.

In the course of this study, the ability to determine pregnancy in an animal with

a history of chronic pregnancy failure led to therapeutic treatment designed to support the pregnancy. The ability to diagnose pregnancy and ovulation using nonstressful, noninvasive methods increases the potential to develop assisted reproduction techniques such as artificial insemination. These noninvasive methods for ovulation and pregnancy detection can also provide a wealth of information about reproductive success in free-ranging rhinoceroses, and possibly shed light on the environmental cues which regulate that success.

CONCLUSIONS

1. Fecal P and E are accurate reflections of circulating P_4 and E_2 during the estrous cycle of the black rhinoceros.

2. Of the behaviors examined, only mounting was associated with physiologic and endocrine indicators of estrus.

3. Physiological changes in the reproductive biology of the black rhinoceros, including estrus and ovulation, are detectable with fecal P and E concentrations.

4. Pregnancy, after day 150 of gestation, can be detected and distinguished from the luteal phase in the black rhinoceros by means of fecal P concentrations.

ACKNOWLEDGMENTS

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