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# Fecal Progesterone, Estrogen, and Androgen Metabolites for Noninvasive Monitoring of Reproductive Function in the Female Indian Rhinoceros, *Rhinoceros unicornis*

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This investigation aimed to establish noninvasive methods for endocrine monitoring of estrous cycles and pregnancy in the Indian rhinoceros. Fecal samples were collected 1–3 times per week from nonpregnant and pregnant captive females ($n = 7$). Enzyme immunoassays for fecal progesterone, androgen, and estrogen metabolites, respectively, were tested for their ability to determine follicular and luteal phases and to characterize endocrine profiles during pregnancy. Antibodies used were raised against pregnanediol (20$\alpha$-OH-pregnanes), 20-oxo-pregnanes, epiandrosterone (17-oxo-androstanes), and total estrogens. Androgens and estrogens were found to be reliable indicators of the follicular phase, whereas 20$\alpha$-OH- and 20-oxo-pregnanes were reliable indicators of luteal function. Progesterone metabolites were also reliable indicators of pregnancy, whereas 17-oxo-androstanes and estrogens were basal throughout gestation. Estrous cycles were regular throughout the year, with an average cycle length of 43.4 ± 1.5 ($n = 27$) days; the length of the follicular phase, as indicated by elevated estrogen levels, was 15.9 ± 1.0 days, whereas the luteal phase, as indicated by elevated 20-oxo-pregnane levels, was 19.1 ± 0.4 days. Fecal pregnane values were already increasing while follicular estrogen values were still decreasing. The length of the diestrus, indicated by basal steroid levels between declining 20-oxo-pregnanes and subsequently increasing estrogens, was 11.4 ± 1.2 days. Pregnane levels increased from the 3rd month of gestation onward and levels exceeded luteal phase concentrations ~10 times by the 7th month of gestation onward. HPLC separation of immunoreactive fecal metabolites indicated the presence of estrone, estradiol-17$\beta$, and several 17-oxo-androstanes, 20$\alpha$-OH-pregnanes, and 20-oxo-pregnanes. Concentrations of a peak with an elution profile similar to that of pregnanediol increased as pregnancy progressed. Postpartum fecal estrogen and 17-oxo-androstane concentrations in one animal indicated follicular development comparable to the follicular phase of the estrous cycle, but this was not followed by a subsequent luteal phase. In conclusion, estrous cycle and pregnancy in Indian rhinoceroses can be monitored using fecal steroid analysis. Pregnane metabolites were reliable indicators of the corpus luteum and pregnancy, whereas fecal 17-oxo-androstanes and estrogens were indicators of the follicular phase. 

*Key Words:* fecal progesterone metabolites; androgens in females; estrogen; noninvasive monitoring; estrous cycle; pregnancy.

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## INTRODUCTION

The five extant species of rhinoceroses include two African species (the black rhinoceras, *Diceros bicornis*, and the white rhinoceros, *Ceratotherium simum*) and three Asian species (the Indian or greater one-horned Asian rhinoceros, *Rhinoceros unicornis*, the Javan or lesser one-horned Asian rhinoceros, *Rhinoceros sondaicus*, and the Sumatran or Asian two-horned rhinoceros, *Dicerorhinus sumatrensis*). The Indian rhinoceros inhabits riverine grasslands and once existed across the northern part of the Indian subcontinent. Now only a few small populations, in eastern India and Nepal, exist. The primary reason for decline has been the loss of habitat and hunting for sport. By the first decade of the 20th century the Indian rhinoceros was almost extinct. Since then the species has been intensely protected and current populations number approximately 1600 rhinoceroses in India and 500 in Nepal. However, poaching for derivatives used in traditional Asian medicines is still estimated to be at a level approximately equivalent to the annual rates of population growth (Foose and van Strien, 1997).

In addition to protection in their natural habitat, managed breeding in zoological institutions is a potential tool for rhinoceros conservation. The current captive population is about 130 individuals. However, this number has not increased since 1991. In addition, the zoo population is based on only 33 founder individuals and 48.4% of genes come from only 3 individuals (Wirz-Hlavacek *et al.*, 1999). To strengthen captive breeding, extension and improvement of appropriate research in reproductive monitoring and evaluation will be required. Information on the reproductive physiology of the Indian rhinoceroses is based mainly on urinary steroid analysis (Kassam and Lasley, 1981; Kasman *et al.*, 1986; Hodges and Green, 1989), whereas in other rhinoceros species data on fecal steroids are also available (Schwarzenberger *et al.*, 1993, 1996b, 1998b; Berkeley *et al.*, 1997; Radcliffe *et al.*, 1997; Garnier *et al.*, 1998; Heistermann *et al.*, 1998; Patton *et al.*, 1999).

Estrous cycle lengths in the Indian rhinoceros were reported to be 43 days (Kassam and Lasley, 1981) and 48 days (Kasman *et al.*, 1986). In contrast, estrous cycles in the black rhinoceros were 25 days (Hindle *et al.*, 1992; Schwarzenberger *et al.*, 1993; Berkeley *et al.*, 1997) and those in the Sumatran rhinoceros were 25 or 21 days (Heistermann *et al.*, 1998; Roth *et al.*, 1998). The white rhinoceros is unusual, as its estrous cycles are either 35 or 70 days in length, and a high proportion (>50%) of captive white rhinoceroses have no estrous cycles at all (Schwarzenberger *et al.*, 1998b; Patton *et al.*, 1999).

The objectives of the present study were to establish new methodological ways to study the reproductive biology of the female Indian rhinoceros. For this purpose group-specific enzyme immunoassays for estrogen, androgen, and progesterone metabolites were used to determine levels of fecal steroid metabolites during the estrous cycle and pregnancy.

## MATERIALS AND METHODS

### *Animals and Sample Collection*

Seven adult female captive Indian rhinoceroses were studied (Bronx NY Studbook No. 066, Dvur Kralove No. 093, Rotterdam No. 185, Munich No. 148 and No. 193; Nuremberg No. 195, Stuttgart No. 204; Wirz-Hlavacek *et al.*, 1999). Animal Nos. 093, 148, 185, 195, and 204 were pregnant and had given birth. Cow No. 148 was pregnant twice, but had aborted during the second half of her pregnancy in 1996 and delivered a stillborn calf in 1999 (possibly due to bacterial uterine infections; R. Hermes, Berlin, pers. comm.).

In general, females were housed individually, but were placed with a male on days of estrus behavior (recorded by keepers). Freshly defecated samples were collected in the morning, two to three times/week, over periods of 6–54 months. Samples were frozen immediately after collection at −20° and shipped to Vienna where they were stored at −20° until analysis.

### *Fecal Extraction*

Extraction of fecal samples was done using an extended version of the methanol extraction method used for several other mammalian species (Schwar-

zenberger *et al.*, 1997), including the black and white rhinoceros (Schwarzenberger *et al.*, 1993, 1996b, 1998b). This elaborated method yielded a cleaner and more concentrated extract (Goymann *et al.*, 1999; Möstl *et al.*, 1999), allowing the determination of low estrogen concentrations during the luteal phase of the estrous cycle. Briefly, 0.5 g of wet feces was mixed with 0.5 ml of water and 4 ml of methanol. Samples were vortexed for 30 min. After centrifugation 1 ml of the methanol was transferred into a new vial, mixed with 0.25 ml of a 5% $NaHCO_3$ solution and 5 ml diethylether, and then vortexed for 30 s. After centrifugation (10 min) the extract was placed at −20° for 30 min. Thereafter, the supernatant ether phase was placed into a new vial, evaporated, and redissolved in 0.5 ml assay buffer, and aliquots were analyzed by enzyme immunoassays (EIA).

### *Enzyme Immunoassays*

Fecal extracts were analyzed by EIAs for immunoreactive progesterone, androgen, and estrogen metabolites. Briefly, the group-specific antibodies used in the EIAs were raised in rabbits and assays included 20-oxo-pregnanes (20-oxo-P) (antibody: 5$\alpha$-pregnane-3$\beta$-ol-20-one 3HS:BSA; Schwarzenberger *et al.*, 1996b), 20$\alpha$-OH-pregnanes (20-OH-P) (5$\beta$-pregnane-3$\alpha$,20$\alpha$-diol 3HS:BSA; trivial name pregnanediol; Schwarzenberger *et al.*, 1993), 17-oxo-androstanes (5$\alpha$-androstane-3,17-dione 3-CMO:BSA; trivial name epiandrosterone; Möstl and Brunner, 1997), and total estrogens (estradiol-17$\beta$-OH 17-HS:BSA; Patzl *et al.*, 1998). Significant cross-reactivities in the progesterone and androgen metabolite assays are those with 5-reduced steroid metabolites and thus results are designated as "pregnanes" and "androstanes," respectively. Several previous publications have shown that these are the principal fecal metabolites of progesterone and testosterone excreted, whereas fecal estrogens are predominantly estradiol or estrone (Palme *et al.*, 1996; Schwarzenberger *et al.*, 1996a, 1997). Assays were validated by demonstrating parallelism between standard curves and serial dilutions of fecal extracts. The intra- and interassay coefficients of variation for these assays were similar to those described previously and were approx 10 and 15%, respectively.

### *High-Performance Liquid Chromatography (HPLC) of Fecal Extracts*

To determine assay specificity and to obtain indications of the possible structure of immunoreactive steroid metabolites, extracts of fecal samples during the follicular and mid-luteal phase and during pregnancy (months 3 and 11) of rhinoceros No. 093 were separated on a straight-phase HPLC system (Schwarzenberger *et al.*, 1996b; Palme *et al.*, 1997; Patzl *et al.*, 1998). Samples were mixed with [$^3$H]steroids, extracted, and separated by HPLC using a solvent gradient of methanol in *n*-hexane/chloroform. Fractions were analyzed in the EIAs, and the HPLC elution profiles of the immunoreactive fecal steroids were compared with those of the [$^3$H]steroids and with different 5$\alpha$- and 5$\beta$-pregnanes and -androstanes. In general, steroids eluted depending on their polarity. Apolar steroids (those with keto groups) eluted prior to steroids with hydroxyl groups. The *n*-hexane/chloroform ratio was (75/25 v/v) for the separation of pregnanes and androstanes and 50/50 (v/v) for estrogens.

### *Data Analysis*

Data are presented as mean ± SE. Estrous cycle length, and calculation of the mean values were based on peak estrogen concentrations, which were designated as day 0. Values were arranged in periods of 15 days before and 30 days after day 0. Because samples were not collected every day, data were combined for 2 days and mean values calculated; this arrangement resulted in 6–30 samples per mean.

The length of the follicular (FP) and luteal (LP) phases of individual reproductive cycles was calculated. Definition of the FP and LP was based on fecal estrogen and 20-oxo-P values, respectively. FP was defined as the first point after estrogen values had increased and remained at >5 ng/g feces; similarly, LP was defined by 20-oxo-P values of >70 ng/g. Because samples were not collected every day, the interval between two consecutive values was divided by 2 and resulting values were added to the FP and the LP. Similar criteria were used to calculate the time between the end of the LP and the beginning of the FP, which was designated as diestrus. The Pearson coefficient of correlation and linear regression were calculated between the concentrations of fecal estrogens

and 17-oxo-androstanes and between 20-oxo-pregnanes and 20$\alpha$-OH-pregnanes.

## RESULTS

### *HPLC Separation of Fecal Immunoreactive Metabolites*

Elution profiles of estrogens in a sample collected during the follicular phase indicated that immunoreactive estrogens in the Indian rhinoceros consist mainly of estrone (about 66%) and estradiol-17$\beta$ (30%), whereas estradiol-17$\alpha$ was only about 3% (Fig 1a). Fecal 17-oxo-androstanes consisted of four immunoreactive metabolites (Fig. 1b). The immunoreactive peak partly coeluting with [$^3$H]androstendione was not considered to be pure androstendione, as the two peaks did not precisely overlap.

HPLC separation of immunoreactive progesterone metabolites detected several immunoreactive 20-oxo-P and 20$\alpha$-OH-P peaks in the samples collected during the LP and pregnancy (Fig 1c). Whereas 20-oxo-P peaks between these reproductive stages were comparable, qualitatively, but not quantitatively, 20$\alpha$-OH-P immunograms were different. Primarily the peak eluting in fraction 48–50 became more prominent as pregnancy progressed; this peak had a polarity comparable to pregnanediol (5$\beta$-pregnane-3$\alpha$,20$\alpha$-diol). Due to lack of standards and GCMS, the exact identities of the other immunoreactive peaks are unknown.

### *Fecal Steroid Profiles during the Estrous Cycle*

A representative profile of fecal immunoreactive 20-oxo-P, 20$\alpha$-OH-P, 17-oxo-androstanes, and estrogens is shown in Fig. 2. In general, fecal estrogens and 17-oxo-androstanes showed similar patterns and were related to estrus behavior. The coefficient of correlation between these two hormones was 0.53 ($n = 303$; $P < 0.001$; 17-oxo-androstanes = 55,99 + (3,14 $\times$ estrogens)). Similar to estrogens and 17-oxo-androstanes, the 20$\alpha$-OH- and 20-oxo-pregnanes showed a matching pattern and were significantly correlated ($r = 0.72$; $n = 310$; $P < 0.001$; 20-oxo-P = 23,86 + (0,43 $\times$ 20$\alpha$-OH-P)). Peak estrogen concentrations varied between animals and between cycles of individual animals (range 13.2–286; median 85 ng/g).

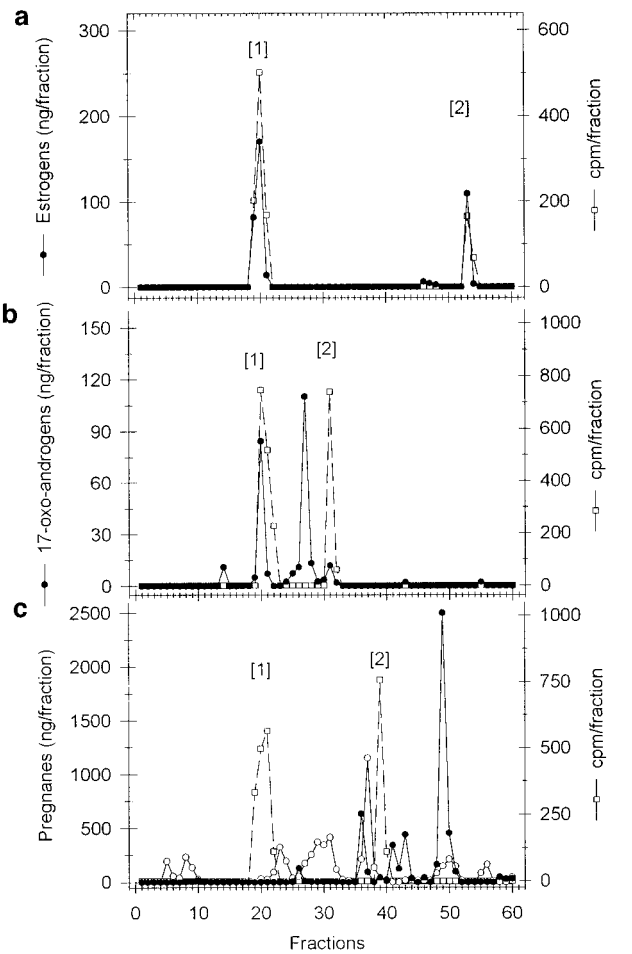


FIG. 1. HPLC separation of immunoreactive steroids in fecal samples of an Indian rhinoceros (No. 093). Depicted results were obtained from samples collected during the follicular phase (a, b) and during the last third of gestation (c). Elution patterns of $^3$[H]steroids (□ cpm/fraction; [1, 2]) were determined by liquid scintillation counting, and immunoreactive steroids were analyzed with EIAs. Concentrations in ng/fraction were calculated for 1 g of feces without correction for methodological losses. (a) Fecal estrogens (●) were compared to elution profiles of $^3$[H]estrone [1] and $^3$[H]estradiol-17$\beta$ [2]; estradiol-17$\alpha$ eluted in fractions 46–48. (b) Fecal 17-oxo-androgens (●) were compared to elution profiles of $^3$[H]androstendione [1] and $^3$[H]testosterone [2]. (c) Fecal 20-oxo-pregnanes (○) and 20$\alpha$-OH-pregnanes (●) were compared to elution profiles of $^3$[H]progesterone [1] and $^3$[H]20$\alpha$-dihydroprogesterone [2]. The 20$\alpha$-OH-P peak eluting in fractions 48–51 had a polarity comparable to pregnanediol, and concentration of this peak increased as pregnancy progressed.

Calculations of estrous cycle profiles were based on results of four animals (nine, nine, five, and four cycles). Mean length of the ovarian cycle, defined as the

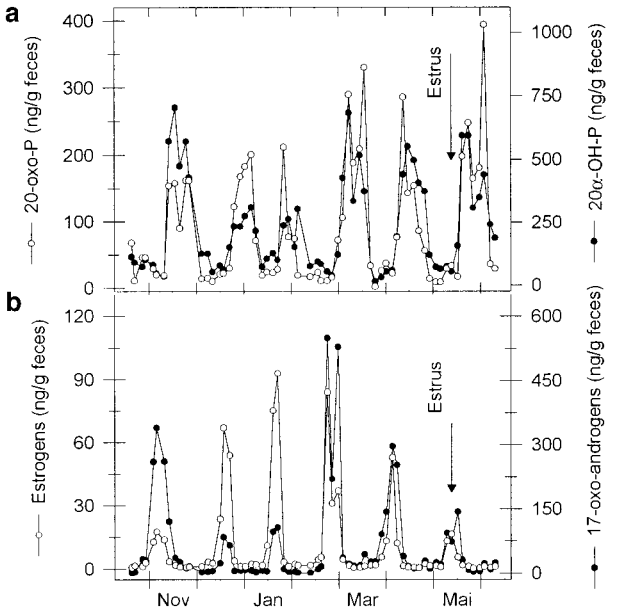


FIG. 2. Profiles of (a) fecal 20-oxo-pregnanes (○) and 20α-OH-pregnanes (●), and (b) fecal estrogens (○) and 17-oxo-androgens (●) during estrous cycles in an Indian rhinoceros (No. 204). Arrows indicate estrus behavior.

interval between two estrogen peaks was 43.4 ± 1.5 days ($n$ = 27; Fig. 3). The duration between the estrogen peak and the following 20-oxo-P peak was 15.4 ± 1.0 days and the time between the 20-oxo-P peak and the succeeding estrogen peak was 28.2 ± 1.3 days. Mean length of FP based on elevated estrogen concentrations was 15.9 ± 1.0 days and mean LP length based on 20-oxo-P was 19.1 ± 0.4 days. Progesterone metabolites began to increase while estrogens were decreasing. Diestrus lasted 11.4 ± 1.2 days.

### *Fecal Steroid Profiles during Pregnancy and Postpartum*

Fecal 20-oxo- and 20α-OH-pregnanes during the 2nd and the beginning of the 3rd month of pregnancy were lower than the midluteal phase values (Fig. 4). Pregnane levels increased from the 3rd month of gestation onward and levels exceeded luteal phase concentrations ~10 times by the 7th month of gestation onward. In contrast, 17-oxo-androstanes and estrogens were basal throughout pregnancy. Pregnancy length was 425, 469, 483, 486, and 487 days.

Between days 29 and 53 of the postpartum period, fecal 17-oxo-androstanes and estrogens in one animal (No. 093) were elevated and indicated follicular development comparable to the follicular phase of the estrous cycle. However, this FP was not followed by subsequent luteal phase elevation in pregnane concentrations, indicating that no ovulation had occurred.

## DISCUSSION

The objective of this study was to characterize long-term fecal steroid hormone excretion during estrous cycles and pregnancy in the Indian rhinoceros. Fecal reproductive monitoring in Indian rhinoceroses is possible using pregnane, estrogen, and androstane assays. The present findings thus confirm and extend previous results on reproductive monitoring in Indian rhinoceroses which were based on urinary steroid hormone analysis (Kassam and Lasley, 1981; Kasman *et al.*, 1986; Hodges and Green, 1989). The general advantage of fecal versus urine samples is easier sample collection; thus, fecal steroid analysis is a promising

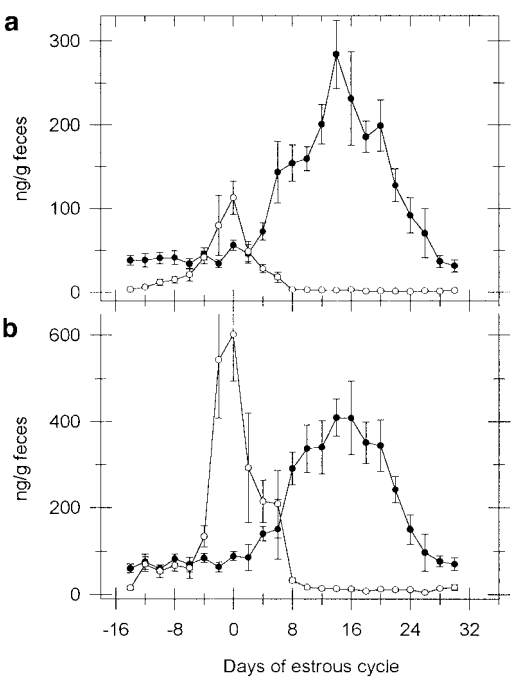


FIG. 3. Composite profiles of (a) fecal estrogens (○) and 20-oxo-pregnanes (●) and (b) fecal 17-oxo-androgens (○) and 20α-OH-pregnanes (●) during estrous cycles in Indian rhinoceroses ($n$ = 4). Data were derived from 27 cycles and are presented as mean ± SE.

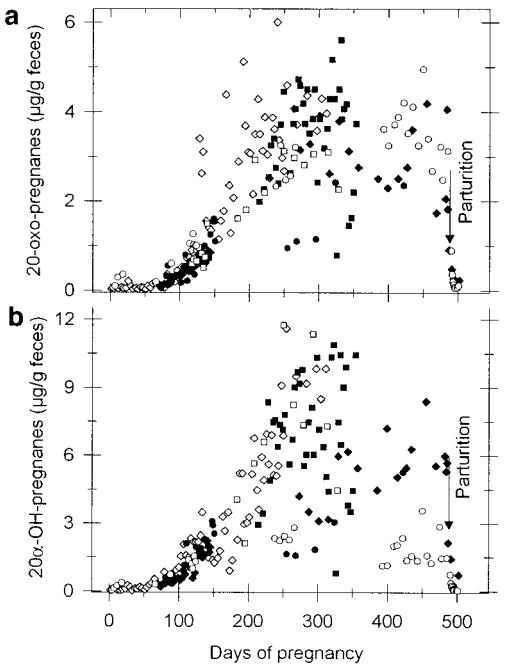


FIG. 4. Concentrations of fecal (a) 20-oxo-pregnanes and (b) 20α-OH-pregnanes in pregnant Indian rhinoceroses: (○) No. 093; (●) No. 185; (□) No. 145 (this pregnancy ended with an abortion); (■) No. 145 (full term pregnancy); (◇) No. 195; and (◆) No. 204.

tool for future fieldwork. Additional advantages in the Indian rhinoceros appear to be pregnancy diagnosis from the 3rd month of gestation onward, whereas urinary steroid analysis allowed this only from mid-pregnancy onward (Kasman *et al.*, 1986; Hodges and Green, 1989). Another beneficial difference between urine and fecal steroid analysis is that in addition to pregnanediol and estrogen assays, which were also used for urine analysis, assays for fecal 17-oxo-androstanes and 20-oxo-pregnanes have now been established.

Fecal 17-oxo-androstanes and estrogens indicated follicular activity in the Indian rhinoceros. Androgens are intermediates in estrogen biosynthesis; however, reports on the excretion of androstanes during the follicular phase of the estrous cycle are available only for carnivores. These studies used the same assay employed in the present study and species studied include the domestic dog (Möstl and Brunner, 1997) and sun bear (Schwarzenberger *et al.*, 1998a). There are no explanations available why some species, including the Indian rhinoceros, excrete 17-oxo-androstanes in rather high quantities into the feces. However, a common feature of the reproductive cycle of the Indian rhinoceros, the sun bear, and the domestic dog is a follicular phase of about 2 weeks in length.

Estrogens have not generally been analyzed in fecal samples of other rhinoceros species. Although Berkeley *et al.* (1998) reported elevated concentrations of fecal estrogens during the follicular phase of nonpregnant black rhinoceroses, there is no general agreement on the measurement of fecal estrogens during the estrous cycles of African rhinoceros species. The assay used for this study did not reliably indicate elevated estrogen levels during follicular phases of the two African rhinoceros species (F. Schwarzenberger, unpublished results). In the Sumatran rhinoceros, radioactive estradiol metabolites were predominantly (nearly 70%) excreted into the urine (Heistermann *et al.*, 1998). Nevertheless, HPLC separation of radioactive fecal estrogen metabolites in this species revealed two peaks, the major one coeluting with estrone and the less prominent one with estradiol-17$\beta$. Fecal immunoreactive estrogens in the Indian rhinoceros thus are comparable to those in the Sumatran rhinoceros. In contrast, in a radiometabolism study in the white rhinoceros, estrone was not found, but both epimers of estradiol accounted for all estrogens detected (Hindle and Hodges, 1990). Baseline levels of estrogens during gestation in fecal samples of Indian rhinoceroses are in agreement with the low levels measured in urine samples (Kasman *et al.*, 1996).

Fecal pregnanes in the present study indicated steroid production from the corpus luteum and the feto placental unit. This is in agreement with several reports on the measurement of fecal progesterone metabolites in African rhinoceroses (Ramsay *et al.*, 1987; Kuckelkorn and Dathe, 1990; Kock *et al.*, 1991; Hindle *et al.*, 1992; Schwarzenberger *et al.*, 1993, 1996b, 1998b; Czekala and Callison, 1996; Berkeley *et al.*, 1997; Radcliffe *et al.*, 1997; Garnier *et al.*, 1998; Patton *et al.*, 1999) and Sumatran rhinoceros (Heistermann *et al.*, 1998). In addition, the presence of several immunoreactive fecal progesterone metabolites is comparable to findings in other mammalian species (Schwarzenberger *et al.*, 1996a, 1997). In contrast to the black and white rhinoceroses, in which 5$\alpha$-reduced pregnanes (5$\alpha$-pregnane-3,20-dione, 5$\alpha$-pregnane-3$\alpha$-ol-20-one, and 5$\alpha$-pregnane-3$\beta$-ol-20-one; Schwarzenberger *et al.*, 1996b, 1998b) appear to dominate in the feces, 5$\beta$-reduced pregnanes like pregnanediol seem to be equally or

even more important in the Indian rhinoceros. The presence of 5$\beta$-reduced pregnane metabolites in the Indian rhinoceros compares with the Sumatran rhinoceros in which the three most abundant progesterone metabolites in a radiometabolism study were identified as 5$\beta$-pregnane-3$\alpha$,20$\alpha$-diol, 5$\beta$-pregnane-3$\alpha$-ol,20-one, and a second pregnanediol, the exact structure of which could not be deduced (Heistermann *et al.,* 1998).

The fecal progesterone metabolite concentrations in the pregnant Indian rhinoceros demonstrate that progesterone metabolite production by the fetoplacental unit can be used for pregnancy diagnosis as in other rhinoceros species (Ramsay *et al.,* 1987; Hodges and Green, 1989; Kuckelkorn and Dathe, 1990; Kock *et al.,* 1991; Schwarzenberger *et al.,* 1993, 1996b, 1998b; Czekala and Callison, 1996; Berkeley *et al.,* 1997; Garnier *et al.,* 1998; Patton *et al.,* 1999). Gestation length in the Indian rhinoceros at ~16 months is slightly longer than the ~15 months reported in the black and white rhinoceroses. Except for one pregnancy of 425 days, other gestation lengths in this study are in agreement with the 476 and 459 days reported by Hodges and Green (1989). Gestation period was truly 425 days in one animal, since animals were placed together only on the day of estrus. Although this pregnancy was appreciable shorter, the female calf was healthy, but had a comparable low birth weight.

The pregnancy profile of urinary pregnanediol–glucuronide (Kasman *et al.,* 1986; Hodges and Green, 1989) was different from those of fecal 20-oxo- and 20$\alpha$-OH-pregnanes. Urinary values were low during the first 2 months of pregnancy and then slightly increased, but a marked increase was not seen before the 7th month of pregnancy. In contrast both, fecal 20-oxo- and 20$\alpha$-OH-pregnanes continuously increased from day 75 of pregnancy until day 300. Thus, the measurement of pregnane metabolites in fecal samples allows pregnancy diagnosis appreciably earlier than the analysis of pregnanediol in urine samples.

In conclusion, monitoring of the estrous cycle and pregnancy in Indian rhinoceroses is possible using fecal steroid analysis. Pregnane metabolites were reliable indicators of luteal and placental function, whereas fecal 17-oxo-androstanes and estrogens were indicators of the follicular phase of the estrous cycle. This study further emphasizes considerable species differences in cycle length and steroid metabolism within the family Rhinocerotidae. Comparison with reports on other species of rhinoceroses revealed that the steroid metabolism in the Indian rhinoceros is more comparable to that of the Sumatran rhinoceros than to that of the African rhinoceros species.

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