

## RESEARCH ARTICLES

# Urinary Steroid Evaluations to Monitor Ovarian Function in Exotic Ungulates: V. Estrogen and Pregnanediol-3-Glucuronide Excretion in the Black Rhinoceros (*Diceros bicornis*)

E.C. Ramsay, L.H. Kasman, and B.L. Lasley

Oklahoma City Zoo, Oklahoma City (E.C.R.); San Diego Zoo Research Department, San Diego Zoo, San Diego (L.H.K.) and Department of Reproduction, University of California, School of Veterinary Medicine, Davis (B.L.L.)

A study of female black rhinoceros (*Diceros bicornis*) urinary steroid and steroid metabolite excretion was performed to determine if techniques useful for monitoring reproductive events in the Indian rhinoceros (*Rhinoceros unicornis*) could be utilized to evaluate the black rhinoceros. Urine samples from 19 zoo-held black rhinoceros were analyzed for estrogen, estrone conjugates (EC), and pregnanediol-3-glucuronide (PDG) content by direct radioimmunoassays. Estrogen analysis revealed that >95% of the estrogens present in female black rhinoceros urine are conjugated, with estrone and estradiol accounting for virtually all of these estrogens. There is no observable difference in the amount of estrogen present in estrus; postestrus; and early-, mid-, and late-gestation urine samples. Analysis of serial urine samples for EC failed to reveal any discernible levels or patterns which reflected reproductive status. Neither nonpregnant nor early-gestational female black rhinoceros' urine samples contained detectable amounts of PDG. Urinary PDG concentrations became measurable in midgestation (9-12 months prior to parturition) and rose steadily throughout the remainder of gestation. PDG levels declined sharply and became nondetectable 1 day postpartum. Though a wide range in PDG levels was observed among individual pregnant animals, each female consistently excreted increasing amounts of PDG through latter pregnancy.

**Key words:** urinary estrogens, urinary hormones, reproduction

## INTRODUCTION

The black rhinoceros (*Diceros bicornis*) population in the wild has recently experienced a precipitous decline owing to illegal hunting [Cummings et al, 1986]. During the same period the captive black rhinoceros population has failed to reproduce in sufficient numbers to maintain itself and, therefore, does not currently represent a safeguard for this species' survival [Linderman, 1984]. If a captive population of black rhinoceros is to be maintained without further recruitment from

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Address reprint requests to Dr. Bill Lasley, Department of Reproduction, School of Veterinary Medicine, University of California, Davis, CA 95616.

the wild, its propagation must be significantly improved and methods for enhancing reproduction in captive animals developed. The ability to evaluate reproductive status and monitor ovarian function would greatly aid the management of captive black rhinoceros reproduction and provide information important to the development of artificial reproduction techniques.

The current study was performed to determine whether urinary hormone assays, which are useful for evaluation of reproductive status in the Indian rhinoceros (*Rhinoceros unicornis*), could be directly applied to the black rhinoceros. Black rhinoceros urine samples representative of major reproductive events were analyzed to determine the primary urinary estrogen and progesterone metabolites in this species. Specifically estrogen and progesterone metabolite excretion patterns during the estrous cycle and pregnancy were evaluated in an attempt to compare the results to similar measurements in related species.

## MATERIALS AND METHODS

Serial urine samples were collected from 19 adult female black rhinoceros held in 14 zoological collections between January 1983 and March 1985. Samples were obtained at least biweekly from all animals. Collection was by either midstream catch or aspiration from the ground and all samples were frozen immediately without preservatives until analysis. Behavioral observations were made by the animals' keepers. Urine samples from two animals which delivered calves during the course of the study were utilized as representative of major reproductive events: estrus samples were collected on days of presumed fertile breedings; postestrus (days 5–10 after fertile breedings); early-gestation (310, 344, and 360 days prior to parturition); midgestation (160, 181, and 222 days prior to parturition), and late-gestation samples (60, 62, and 70 days prior to parturition). All pregnancies in the study were confirmed by parturition.

### Creatinine Analysis

Urine samples were analyzed for creatinine (Cr) content by the technique of Taussky [1954] to evaluate individual sample quality and to index hormone values for comparison. All hormone measurements are presented as mass/mgCr. Samples containing less than 0.1 mgCr/ml were deemed too dilute for accurate hormonal evaluation.

### Estrogen Component Analyses

Selected urine samples were evaluated for free (not conjugated) and total (free plus conjugated) estrogen immunoreactivity. An aliquot (0.5 ml) of each sample was extracted with 5 ml ether, and the extractant was assayed for free estrogens using a nonspecific estrogen radioimmunoassay (RIA) described by Czekala et al [1981]. Separate aliquots of each sample were analyzed for total estrogen immunoreactivity by hydrolyzing the sample with  $\beta$ -glucuronidase/aryl-sulfatase, extracting with ether, and assaying the extractant with the total estrogen RIA.

Conjugated estrone (E1) and estradiol (E2) were evaluated by subjecting the aqueous residual of ether extracted samples (0.5 ml) to enzyme hydrolysis, reextraction with ether, and separation by celite column chromatography [Anderson et al, 1976]. Tritiated E1 and E2 (1,000 cpm, each) were added to each sample to serve as chromatographic markers and to monitor methodological losses prior to the second extraction. The chromatography eluates were divided and the aliquots assayed for

free E1 and E2 using the total estrogen RIA and E1 and E2 tracers and standards, respectively. An aliquot of each celite column eluate was also evaluated for recovery of tritiated markers.

Confirmation of the estrogen components present in samples was achieved by high-performance liquid chromatography (HPLC). Aliquots (0.5 ml) of these samples were enzyme hydrolyzed, and tritiated markers were added (E1, E2, and estriol; 1,000 cpm each). The hydrolyzates were extracted with ether and the organic residuals separated by HPLC as described by Kassam and Lasley [1981]. An aliquot of each eluate was assayed using the total estrogen RIA and an aliquot of each eluate evaluated for recovery of the tritiated markers.

A second hydrolysis method, ammonolysis [Bain et al, 1984], was also applied to representative samples in an attempt to free possible estrogen conjugates not hydrolyzed by the enzyme. Paired aliquots (0.5 ml, 3 pairs each) of estrus and postestrus urine samples were hydrolyzed by enzyme or ammonolysis, extracted with ether, and assayed using the total estrogen RIA. To validate the ammonolysis methodology in the black rhinoceros, 100 ng of estradiol 3,17 disulfate was added to matched aliquots of the samples and those aliquots were processed in the identical manner described above.

### **Estrone Conjugate Analysis**

Serial urine samples were analyzed for immunoreactive estrone conjugates (EC) with the direct radioimmunoassay (RIA) of Shideler et al [1983], with the EC antisera produced by Kasman et al [1986]. The interassay coefficient of variation for the EC assay was 6.5% at 28–31% binding (n=29). HPLC was used to confirm the identity of the estrone conjugates present in selected samples. Samples were evaluated by the HPLC method described by Shideler et al [1983] and matched samples assayed directly (without HPLC) for EC immunoreactivity to compare with concentrations of EC found in HPLC eluates. Serially diluted samples were analyzed to evaluate parallelism between glucuronide standards and to determine the appropriate volume to assay.

### **Pregnanediol-3-Glucuronide Analysis**

Urinary pregnanediol-3-glucuronide (PDG) analysis was performed on serial samples from each animal following the protocol of Loskutoff et al [1982]. The interassay coefficients of variation for the PDG assay were 14.5 and 15.0% at 73–76% and 29–31% binding, respectively (n=21). Aliquots of postestrus and midgestation samples were subjected to HPLC to identify PDG-immunoreactive substances [Kasman et al, 1986]. Serially diluted postestrus and midgestation samples were also evaluated for dose-response parallelism to serially diluted pure PDG standards.

## **RESULTS**

### **Component Estrogen Evaluations**

Analysis of estrogen evaluations are presented in Table 1. Total estrogen analysis of ether extracts of untreated versus hydrolyzed samples indicate that >95% of the estrogens present in female black rhinoceros urine are conjugated. Enzyme hydrolysis and celite column chromatography of aqueous residuals identified quantifiable amounts of E1 and E2 in all samples. Levels of enzyme hydrolyzable E1 or E2 varied little with respect to reproductive state and the sum of E1 and E2 in each

**TABLE 1.** Total estrogens (samples assayed following enzyme hydrolysis), free estrogens (samples assayed without enzyme hydrolysis), estrone and estradiol (samples assayed following enzyme hydrolysis and celite chromatography) immunoreactivity ( $\bar{x} \pm \text{SEM}$ ) in female black rhinoceros urine samples from major reproductive events expressed as ng hormone/mg creatinine

	Estrus <sup>a</sup>	Post- estrus <sup>b</sup>	Gestation		
			Early <sup>c</sup>	Mid <sup>d</sup>	Late <sup>e</sup>
Total estrogens	43.8 ± 4.2	41.8 ± 4.3	43.2 ± 3.8	38.5 ± 7.7	43.6 ± 4.2
Free estrogens	0.52 ± .14	0.54 ± .06	0.52 ± .16	0.38 ± .24	0.47 ± .18
Estrone (E1)	23.7 ± 4.9	21.2 ± 2.3	22.9 ± 2.9	16.7 ± 10.4	19.4 ± 5.4
Estradiol (E2)	21.64 ± 3.5	20.7 ± 2.3	13.3 ± 7.0	18.7 ± 3.9	24.7 ± 4.0
E1 + E2	45.4 ± 6.7	41.9 ± 1.11	42.8 ± 4.6	39.7 ± 9.0	44.0 ± 1.5
E1/E2	1.10	1.02	1.72	0.89	0.79
n	5	5	3	3	3

<sup>a</sup>Days of presumed fertile breedings.

<sup>b</sup>Days 5 to 10 after presumed fertile breedings.

<sup>c</sup>Days 310, 344, and 360 prior to parturition.

<sup>d</sup>Days 160, 181, and 222 prior to parturition.

<sup>e</sup>Days 60, 62, and 70 prior to parturition.

sample, as measured separately following celite chromatography, was highly correlated ( $p=0.98$ ,  $n=19$ ) to the total estrogen immunoreactivity.

The HPLC elution patterns of immunoreactive E1 and E2 corresponded to the elution patterns of tritiated E1 and E2 and revealed these to be the only immunoreactive substances measured by the total estrogen RIA. Analysis of samples hydrolyzed by ammonolysis, from estrus and postestrus periods, failed to reveal an increase in estrogen levels as compared to enzyme hydrolysis. In samples to which estradiol disulfate was added, 87% was measurable as free E2 following ammonolysis, whereas enzyme hydrolysis of samples to which estradiol disulfate was added revealed little additional immunoreactivity over pretreatment estradiol levels.

### Estrone Conjugate Evaluations

Results of estrone conjugate (EC) analyses are presented in Table 2. Dose-response dynamics and HPLC chromatography confirmed the identity of estrone-3-sulfate and estrone-3-glucuronide in all samples. Estrone-3-glucuronide levels averaged  $71.6\% \pm 3.5\%$  ( $\bar{x} \pm \text{SEM}$ ,  $n=15$ ) of the total EC immunoreactivity. Compared to matched samples assayed without prior chromatography, the sum of the two estrone conjugate components averaged  $97.0\% \pm 0.9\%$  ( $n=15$ ) of the total EC immunoreactivity. Analysis of serial weekly urine samples from nonpregnant and pregnant black rhinoceros females with the EC RIA ( $n=1,450$ ) failed to reveal any discernible levels or patterns which reflected the estrous cycle or pregnancy.

### Pregnanediol-3-Glucuronide Evaluations

Dose-response dynamics of postestrus urine samples produced a nonparallel curve to PDG standards and HPLC revealed no PDG in postestrus urines. Furthermore, no measurable levels of urinary PDG immunoreactivity were observed in any nonpregnant sample analyzed. In contrast, urine samples from midgestation produced a parallel dose response to PDG standards and HPLC revealed the presence of immunoreactivity cochromatographing with the tritiated PDG marker.

**TABLE 2.** Estrone conjugate immunoreactivity ( $\bar{x} \pm \text{SEM}$ ) in female black rhinoceros urine samples from major reproductive events expressed as ng hormone/mg creatinine

	Estrus <sup>a</sup>	Post estrus <sup>b</sup>	Gestation		
			Early <sup>c</sup>	Mid <sup>d</sup>	Late <sup>e</sup>
Estrone conjugates	16.4 ± 1.6	15.5 ± 1.6	15.3 ± 0.9	18.8 ± 1.7	18.1 ± 2.2
Estrone glucuronide (EG)	12.6 ± 1.3	10.9 ± 3.0	10.9 ± 1.5	14.9 ± 1.5	14.9 ± 0.9
Estrone sulfate (ES)	4.9 ± 0.4	5.4 ± 0.9	3.6 ± 0.6	5.1 ± 0.7	5.8 ± 0.3
EG + ES	17.6 ± 1.2	16.7 ± 1.8	14.5 ± 1.8	19.1 ± 1.8	20.6 ± 0.9
EG/ES	2.6	2.0	3.0	2.8	2.6
n	3	3	3	3	3

<sup>a</sup>Days of presumed fertile breedings.

<sup>b</sup>Days 5 to 10 after presumed fertile breedings.

<sup>c</sup>Days 310, 344, and 360 prior to parturition.

<sup>d</sup>Days 160, 181, and 222 prior to parturition.

<sup>e</sup>Days 60, 62, and 70 prior to parturition.

Figure 1 illustrates the urinary PDG values from eight pregnancies of six black rhinoceros, plotted in respect to the day of parturition. PDG levels rose from nondetectable to measurable between 9 and 12 months prior to parturition. Though a wide range of values was found among individual animals, each animal consistently excreted increasing amounts of PDG through gestation. PDG levels declined sharply to nonmeasurable levels one day postpartum and remained low through the last sample analyzed (40 days postpartum).

In five pregnancies where breeding dates were known, the interval between the date of last breeding and parturition ranged from 389 to 480 days. The animal with the 389-day interval had also been bred at days 460, 467, and 493 prior to parturition. Multiple breeding dates were observed in two other females; one animal was bred 438 and 511 days prior to parturition and the other was bred on days 480, 505, and 555 prior to parturition.

## DISCUSSION

Analyses of female black rhinoceros' urine indicates that the majority of estrogens present are excreted in a conjugated form and that enzyme hydrolysis is capable of hydrolyzing all estrogen components. Conjugated E1 and E2 appear to account for virtually all of these estrogens. Estrone conjugates are present in measurable quantities throughout the estrous cycle and pregnancy; however, neither their levels nor their serial excretion patterns appear to reflect either of these reproductive events.

PDG, in contrast, is not excreted in measurable quantities in urine of nonpregnant or early-gestational female black rhinoceros. The consistent finding of urinary PDG, beginning in midgestation and continuing through the last trimester of gestation, indicates that PDG analysis is potentially useful for the diagnosis of pregnancy in this species. While the range of urinary PDG levels excreted among individuals is wide, gradually increasing amounts through latter gestation is a consistent finding in all animals monitored and suggests that serial PDG evaluation may also be used to monitor fetal/placental viability through the second half of gestation. It is very likely that improved techniques for measuring progesterone metabolites could allow earlier detection of pregnancy and perhaps even luteal function.

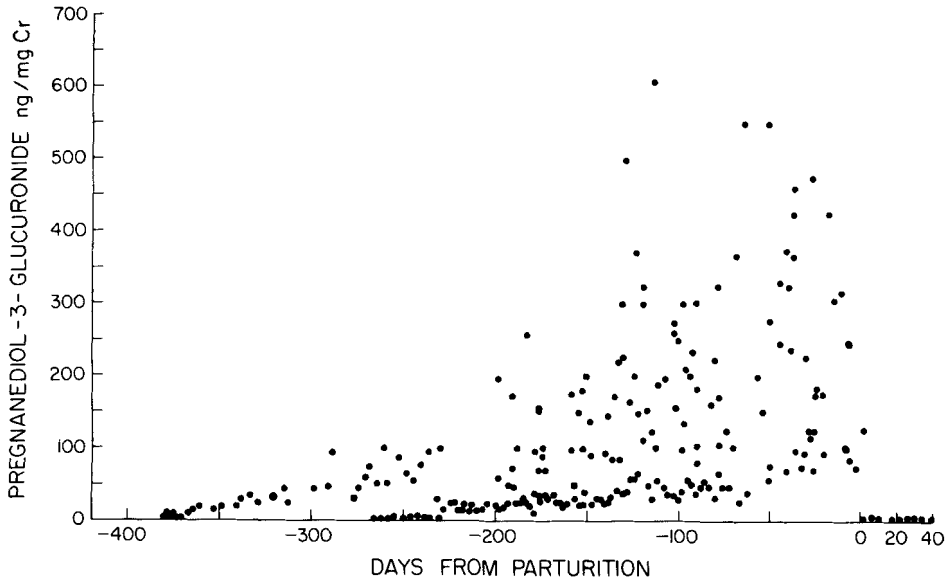


Fig. 1. Immunoreactive urinary pregnanediol-3-glucuronide (PDG) values, indexed by creatinine, from eight gestations of six captive black rhinoceros females, aligned to day of parturition (day 0).

The levels and patterns of EC and PDG excretion in the black rhinoceros differ markedly from those of the Indian rhinoceros, the only other rhinoceros species whose urinary steroid excretion has been reported [Kasman et al, 1986]. The female Indian rhinoceros excretes almost 1,000-fold greater amounts of urinary estrogen, primarily conjugated estrone, during the estrous cycle than the black rhinoceros female; and the dynamics of EC excretion appear to reflect ovarian follicular activity in the Indian species. PDG is found in measurable quantities throughout the Indian rhinoceros estrous cycle and pregnancy, and its measurement can be used for monitoring corpus luteum activity and gestation, following the first luteal phase postconception. As in the black rhinoceros, gradually increasing levels of urinary PDG are seen in the Indian rhinoceros through mid- and late gestation. A precipitous drop in urinary PDG at or before parturition is also seen in both species [Kasman et al, 1986].

In both the black and Indian rhinoceros females urinary EC does not appear to reflect early or late gestation. In contrast, other Perissodactyls' EC excretion increases during pregnancy. In the domestic horse, increasing levels of EC are excreted beginning between 37 and 45 days postconception [Evans et al, 1984]. Urinary EC can also be used to detect pregnancy in the Przewalski horse, *Equus przewalskii* [Boyd, 1987], and in two tapir species [Kasman et al, 1985].

The delay of immunoreactive PDG excretion during pregnancy in the black rhinoceros may reflect a midgestational shift in progesterone metabolism or secretion from the ovaries to the placenta, similar to what occurs in the horse [Ginther, 1979]. This concept is supported by the lack of measurable urinary PDG in nonpregnant black rhinoceros. Interestingly, while urinary PDG is measurable during gestation in the black and Indian rhinoceros, it is not detected during gestation in the horse or tapir [Kasman, unpublished data].

The gestation lengths observed in the present study, based on date of last observed breeding to parturition, demonstrate an unusually large range. If the one

animal's breeding which occurred 389 days preparturition is assumed to be a postconception or spurious breeding, then the lengths of gestation in the five females studied for whom breeding dates are known range from 438 to 480 days ( $\bar{x}$ =462 days). This is similar to the range of previously reported captive black rhinoceros gestations, 416 to 469 days [Greed, 1967; Dittrich, 1967; Yamamoto, 1967; Hays, 1967; Krishna Gowda, 1967]. This previously reported range is also large and includes shorter gestations than those observed in the present study, which further suggests that postconception breedings may have been observed.

The cause of the wide range of PDG levels observed among the eight gestations monitored is unknown. Variation in sample quality, such as time and method of collection and differences in sample storage, is a possible explanation. More likely, these values reflect variation in individual animals' steroid metabolism and excretion. As in the Indian rhinoceros, serial monitoring is required to verify the accuracy of PDG evaluations during the black rhinoceros gestation.

Since urinary EC and PDG analyses do not represent methods by which ovarian function in the black rhinoceros can be monitored, techniques must still be developed to provide captive rhinoceros managers with the information and methods needed to improve captive propagation. Free and conjugated urinary E2 levels should be evaluated to determine if these might better reflect ovarian follicular activity. The black rhinoceros may excrete the majority of its steroids in the feces, and fecal steroid and steroid conjugate analyses may be useful to evaluate and monitor reproductive function in this species.

While the results of the present study are somewhat disappointing with respect to providing techniques for use by rhinoceros managers, considerable information about this species and its reproductive hormone metabolism has been acquired. Urinary total estrogen excretion and EC and PDG levels have been characterized. Urinary EC and PDG can be ruled out as methods for evaluating ovarian activity of the nonpregnant female black rhinoceros, but urinary PDG analysis does appear to be of value for the diagnosis and monitoring of pregnancy. This capability has not been previously available for this species and can potentially aid in the management and movement of individuals. Additional data has been added to the length of black rhinoceros gestation in captivity, and a presumed postconception breeding, a suspected event in this species, has been reported. This suggests that in captivity conception should not necessarily be assumed to occur at the last observed breeding. The present study revealed a marked difference between the black and Indian rhinoceros' urinary steroid conjugate excretion and reinforces the concept that in each new species studied methodologies should be validated and novel techniques may be required.

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Dr. Ramsay's current address is 1018 Glen Brook Avenue, San Jose, CA 95125.

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