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Use of Salivary Steroid Analyses to Assess Ovarian Cycles in an Indian Rhinoceros at the National Zoological Park

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Saliva samples were collected from a female Indian rhinoceros (*Rhinoceros unicornis*) housed at the National Zoological Park, and ether-extracted for analyses of androgen, estrogen, and progestin metabolites to assess ovarian cycles. Analyses of both salivary androgens and estrogens were found to reliably monitor follicular activity. Although the temporal patterns of the two steroids were significantly correlated ($r = 0.62$; $P < 0.05$), they differed slightly. Salivary androgens increased earlier during the follicular phase, although both peaked at the same time in association with behavioral estrus. Based on salivary androgen profiles, the mean duration of the follicular phase was 11.4 days (ranged = 7–15 days; n = 17 cycles). Estrous cycle length, as measured by the time between consecutive androgen peaks, was 47.4 ± 3.4 days (range = 37–86 days). Salivary progestin measurements were effective for monitoring luteal function. The mean duration of the luteal phase was 15.5 ± 1.5 days (range = 10–23 days). In sum, assays were identified for measuring salivary steroids to assess ovarian function in Indian rhinoceroses. However, not all of the assay systems tested were effective, perhaps because of interfering matrix effects. Mate introductions in the Indian rhinoceros often require careful monitoring, and a technique for monitoring hormones in saliva could be an alternative to urine or fecal analyses for identifying estrus and timing breeding in this species. Such a technique would be particularly useful for situations in which it is difficult to collect uncontaminated urine and feces. Zoo Biol 23:501–512, 2004. © 2004 Wiley-Liss, Inc.

Key words: hormones; estrus; androgens; estrogens; progestins

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INTRODUCTION

Protection of the Indian rhino (*Rhinoceros unicornis*) has brought it back from near extinction in the early 20th century to a current population of over 2,400 individuals [Foose, 2002]. However, threats to rhino survival in the wild still exist, and captive populations are small and not increasing [Schwarzenberger et al., 2000]. Thus, captive propagation is all the more important given the uncertainty about the future viability of ex situ rhino populations [Patton et al., 1999]. Our understanding of the reproductive physiology of rhinoceroses in general has increased markedly in recent years, thanks primarily to the development of noninvasive urinary and fecal steroid analyses to assess estrous cyclicity and pregnancy. Studies using urinary steroid metabolite monitoring have been conducted in Indian [Kassam and Lasley, 1981; Schwarzenberger et al., 2000], black [Berkeley et al., 1997; Hindle et al., 1992; Schwarzenberger et al., 1993], and Sumatran [Heistermann et al., 1998; Roth et al., 1998] rhinoceroses. Fecal hormone analyses have been conducted in Indian [Kassam and Lasley, 1981; Schwarzenberger et al., 2000], black [Berkeley et al., 1997; Hindle et al., 1992; Schwarzenberger et al., 1993; Brown et al., 2001], white [Patton et al., 1999; Schwarzenberger et al., 1998; Brown et al., 2001], and Sumatran [Heistermann et al., 1998; Roth et al., 2001] rhinoceroses.

Interest in breeding a genetically valuable Indian rhinoceros pair at the National Zoological Park (NZP) prompted efforts to document the female's reproductive cycles to aid in the proper timing of mate introductions. However, she typically urinated and defecated in a single mass, so it was not possible to collect uncontaminated samples on a routine basis. Therefore, an alternative method based on analysis of salivary hormones was developed to assess ovarian activity.

Salivary monitoring has been used to characterize ovarian and testicular steroidogenic activity in humans and other mammalian species [Atkinson et al., 1999; Cook, 2002; Czekala and Callison, 1996; Ellison, 1993; Fenske, 1996; Ishikawa et al., 2002; Lu et al., 1997; Pietraszek and Atkinson, 1994; Theodorou and Atkinson, 1998; von Engelhardt et al., 2000], and to diagnose pregnancy in black rhinoceroses [Czekala and Callison, 1996]. In this paper we describe the validation of salivary androgen, estrogen, and progestin metabolite analyses for monitoring follicular and luteal function in the female Indian rhinoceros.

MATERIALS AND METHODS

Animal History and Husbandry

The pair of Indian rhinoceros at NZP were maintained in separate enclosures, each of which had indoor-outdoor access and an outdoor cement pool. The animals were fed a pelleted vitamin-mineral-protein supplement two times daily, and fruits and vegetables once daily. Grass hay and water were provided ad libitum, and alfalfa flakes were offered occasionally as a supplement. The female (Mechi, SB#0138) was wild-born in the Royal Chitwan National Park, Nepal, in 1986, and was presented as a gift from the royal family of Nepal to the NZP in 1987. She produced a stillborn calf in 1992, and a live female calf in 1996 sired by Pandu (SB#0101). Both calf and sire have since been transferred to other facilities. The male (Mohan, SB#0049) was wild-born in the Royal Chitwan National Park in 1969, and was transferred from the Miami Metro Zoo to the NZP in 1998. He has never successfully bred, and is

considered a highly aggressive and unpredictable male. Thus, concerns about the safety of the female prompted us to document her estrous cyclicity to ensure that breeding introductions were correctly timed.

At the NZP, an operant conditioning program is utilized to facilitate basic husbandry and medical procedures, and offer stimulation and enrichment to the rhinos. Training for daily saliva collection was achieved by modifying previously learned behaviors. The female was trained to hold her mouth open and remain in position while a plastic measuring spoon was used to collect about 5 ml of saliva from her lower lip. The samples were collected 5–7 times a week for 27 months and stored frozen (at –20°C) in 12 × 75 mm plastic tubes until they were analyzed.

Behaviors characteristic of estrus, such as pacing, blowing, head-tossing, lifting of the tail, vulvar winking, and urine-squirting were recorded in a daily log. Food intake, swelling of the vulva, and presence of vaginal discharge also were recorded. The date of peak behavioral estrus was estimated for each cycle based on observations by the lead keeper.

Salivary Extraction and Steroid Analysis

Saliva samples were ether-extracted and concentrated four times for all hormonal analyses. Prior to extraction, the samples were thawed and mixed, and debris was allowed to settle to the bottom of the tube. For each sample, 1 ml of saliva was placed in a 16 × 125 glass tube. Then 5 ml of fresh diethyl ether (Fisher Scientific Co., LLC, Pittsburgh, PA) was added, and the samples were vortexed for 1 min. The samples were frozen in liquid nitrogen, and the ether fraction was collected in another 12 × 75 glass tube. The samples were frozen again if two noticeable phases were present after the first extraction. The combined ether fraction was air-dried, reconstituted in 1 ml of methanol (Fisher Scientific), and sonicated for 5 min. The methanol fraction was air-dried and then reconstituted in 250 μl of phosphate-buffered saline (PBS) (0.1 M PO_4, 0.14M NaCl, 0.1% bovine serum albumin, pH 7.0).

Androgen metabolites were quantified in salivary extracts by enzyme-immunoassay (EIA) with the use of a polyclonal antiserum (R156/7; 1:7,500 dilution; provided by C. Munro, University of California–Davis, Davis, CA), horseradish peroxidase conjugated label (1:15,000 dilution; C. Munro), and testosterone standards (2.3–600 pg/well; Steraloids, Wilton, NH). Extracted samples (50 μl/well) were added to microtiter plates (Nunc™ Maxisorp™ plates; Nalge Nunc International, Rochester, NY). The known cross-reactivities for the testosterone EIA were as follows: testosterone 100%, 5α-dihydrotestosterone 57.4%, and androstenedione 0.27% (C. Munro, personal communication). The assay sensitivity was 4.5 pg/well.

Estrogen metabolites were quantified with the use of an estradiol EIA kit developed for unextracted human saliva (Salimetrics LLC, State College, PA). However, extracted, concentrated saliva had to be used because the samples that were processed according to the manufacturer's recommendations (i.e., freeze/thawing, vortexing, and centrifuging samples only) failed to produce reliable results. The antibody cross-reactivities provided by the manufacturer were as follows: estradiol 100%, estriol 0.08%, estrone 0.263%, testosterone 0.005%, and prednisone 0.006%. The assay sensitivity was 1 pg/ml. Attempts to measure salivary estrogens using the following assay methods were unsuccessful (i.e., serial dilutions of

extracted, concentrated saliva samples did not produce parallel displacement curves): 1) solid-phase estradiol ^{125}I radioimmunoassay (RIA) (Diagnostic Products, Corp., Los Angeles, CA), 2) double-antibody estradiol ^{125}I RIA (ICN Biomedicals, Costa Mesa, CA), 3) total estrogen ^{125}I RIA (ICN Biomedicals); 4) estradiol EIA (antibody R4972; C. Munro), and 5) estrone sulfate EIA (antibody R583; C. Munro).

Progestin metabolite immunoactivity was quantified with the use of a progestin EIA kit specifically developed for unextracted human saliva (Salimetrics LLC). However, again the rhino saliva had to be extracted and concentrated before the analysis was performed. The antibody cross-reactivities stated in the kit literature were: progesterone 100%, 5-pregnadione 2.2%, 5-pregnandione 7%, 20-dihydro-progeterone 1.5%, corticosterone 1.3%, 17α-OH-progesterone 1.9%, testosterone 0.3%, and 17α-OH-pregnenolone 0.1%. The assay sensitivity was 10 pg/ml. Three progestin assays failed to produce a parallel displacement curve from serially diluted, concentrated salivary extracts: 1) an EIA using a broad spectrum progestin antibody (Quidell Clone 425; C. Munro), 2) a pregnanediol-glucuronide (PdG) EIA (antibody P26; C. Munro), and 3) a commercial solid phase progesterone ^{125}I RIA (Diagnostic Products Corp.).

Serial dilutions of concentrated saliva extracts gave displacement curves parallel to the standard curves for each of the assays used to document ovarian activity in this study (testosterone EIA, $r = 0.988$; Salimetrics estradiol EIA, $r = 0.993$; Salimetrics progesterone EIA, $r = 0.997$). The intra- and interassay coefficients of variation for these assays were $< 10\%$ and $< 15\%$, respectively.

High-Performance Liquid Chromatography (HPLC)

Pooled saliva samples during estrus ($n = 6$) and the luteal phase ($n = 6$) were subjected to HPLC analysis to determine androgen and progestin metabolite immunoreactivity, respectively. The samples were extracted as described above; however, once they were resuspended in 1 ml of methanol, the samples were combined into two respective tubes (one pool each for the luteal and estrous periods), air-dried, and then resuspended in 500 μl of PBS buffer, sonicated for 5 min, and vortexed for 1 min.

The sample representing estrus was spiked with ~7,000 dpm of ^{3}H testosterone, aldosterone, androstan-3α-17β-diol, andro-4-ene-3, 17-dione, and DHT for androgen immunoactivity determination, and the luteal phase sample was spiked with ~7,000 dpm of ^{3}H progesterone. Before performing the HPLC, we processed the salivary extracts through a Spice™ C18 sample preparation cartridge (Analtech, Inc., Newark, DE) by priming the cartridge (3 ml of methanol followed by 3 ml of distilled water), loading 500 μl of salivary extract diluted in PBS onto the cartridge, and pushing 5 ml of distilled water through the cartridge, followed by 5 ml of methanol to elute the extract. The methanol portion was collected into a 12 × 75 mm glass tube, and dried under air, and the residue was resuspended in 300 μl of methanol. For the HPLC, 50 μl of the reconstituted sample was injected onto the column (Reverse Phase Microsorb™ MV 100 C18, 5 μm diameter particle size; Varian Analytical Instruments, Woburn, MA). For androgens, a mobile phase of 45% acetonitrile in water over 80 min at room temperature was used at a flow rate of 1 ml/min. For progestins, a multistep gradient mobile phase of 20%, 32%, 50%, and 100% acetonitrile in water over 0, 15, 45, and 60 min at room temperature at a flow

rate of 1 ml/min was used. Effluent samples were collected at 1 ml/tube. To determine the retention times of the ^{3}H reference standards, aliquots (50 μl) from each fraction were counted for radioactivity. The remainder of the fraction was dried down, resuspended in 250 μl of PBS buffer, and analyzed to evaluate immunoreactivity.

Data Analysis

Because the androgen EIA was the first assay shown to correlate with behavioral estrus, and this analysis was conducted over the longest time period (27 months), estrous cycle data were based on this measurement. The beginning of the follicular phase was designated visually as the point at which androgen levels rose above 50 pg/ml for more than 2 consecutive days. The luteal phase began after androgen concentrations fell to baseline after an estrual peak and progestin concentrations began to increase, and the luteal phase ended when progestin concentrations decreased to levels measured during the previous follicular phase.

A Pearson correlation analysis was used to determine the relationship between androgen and estrogen immunoassay data. Average data are presented as the mean ± standard error (SEM).

RESULTS

The elution profiles of androgens in saliva samples subjected to HPLC analysis are shown in Fig. 1. The major immunoreactive peak coeluted with androstandiol

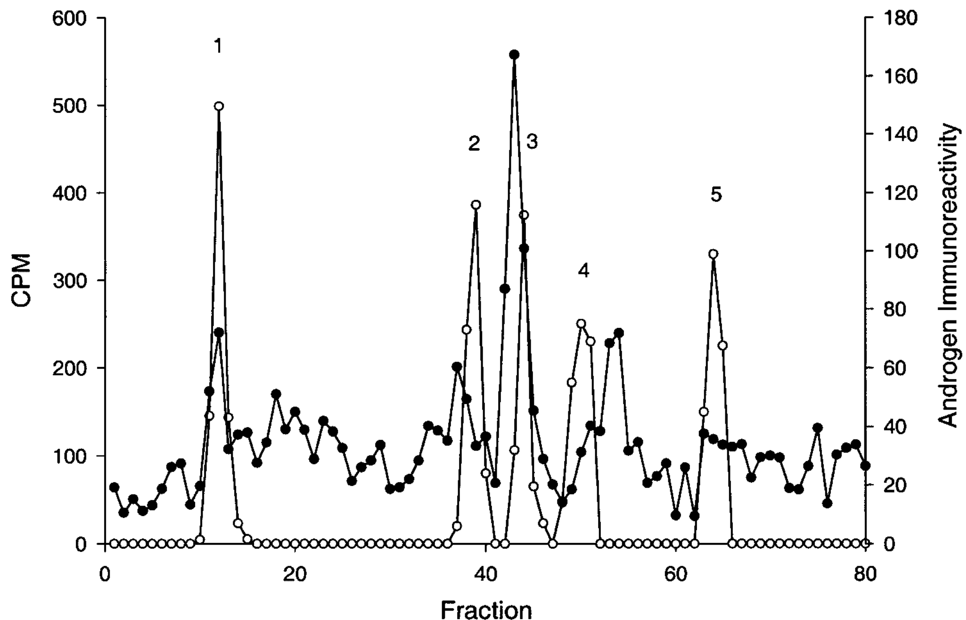

Fig. 1. Cochromatography of estrous phase saliva, demonstrating HPLC separation of immunoreactive androgens. Closed circles represent immunoreactive compounds, and open circles represent radiolabeled compounds. Salivary androgens were compared with the elution profiles of radioactive aldosterone (1), testosterone (2), androstandiol (3), androstenedione (4), and DHT (5).

(androstan-3α-17β-diol), and a second immunoactive peak coeluted with aldosterone. Two unidentified peaks eluted just before and just after the reference tracers for testosterone and androstenedione, respectively. HPLC separation of progestin metabolites produced several immunoactive peaks (Fig. 2), one of which coeluted with the progesterone reference tracer.

We quantified salivary androgen concentrations over a 27-month period for 17 cycles (Fig. 3), salivary estradiol over a 5-month period for 3.5 cycles (Fig. 4), and salivary progestins over a 17-month period for nine cycles (Fig. 5).

Androgen concentrations increased gradually to a peak, and then decreased back to baseline (Figs. 3 and 4). Androgens were elevated for an average of 6.6 days before (range = 2–10 days, median = 7 days) and 4.5 days after (range = 2–9 days, median = 4 days) the estrual peak. The mean duration of the entire follicular phase was 11.5 $\pm$ 0.8 days (range = 7–15 days, median = 12 days). Cycle length, as measured by the time between consecutive androgen peaks, was 47.4 $\pm$ 3.4 days (range = 37–86 days, median = 44 days). Assessment of cycles by salivary estrogens yielded similar results, and the correlation between the two hormones was significant ($r = 0.62$; $n = 129$, $P < 0.001$). However, the temporal patterns differed slightly. Salivary androgens increased gradually from the late luteal phase throughout the follicular phase, and then fell sharply after the peak. By contrast, salivary estrogens were elevated for only 2–3 days before the peak, after which concentrations declined sharply similar to that observed for androgens. Thus, in general, salivary androgens increased about 3 days earlier than the estrogens, although the peaks in the two

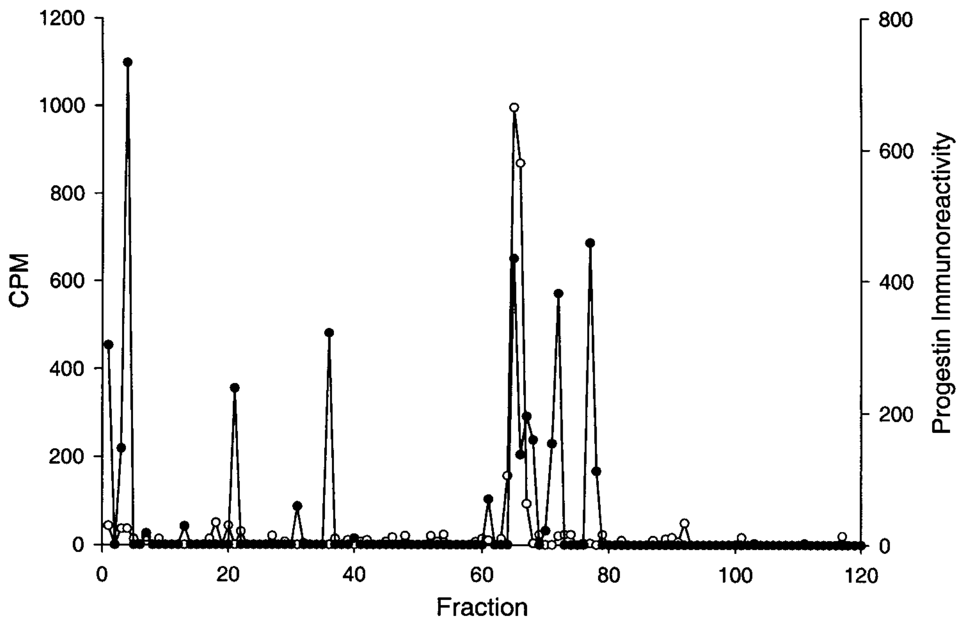

Fig. 2. Cochromatography of luteal phase saliva, demonstrating HPLC separation of immunoreactive progestins. Closed circles represent immunoreactive compounds, and open circles represent radiolabeled compounds. Salivary progestins were compared with the elution profile of radioactive progesterone.

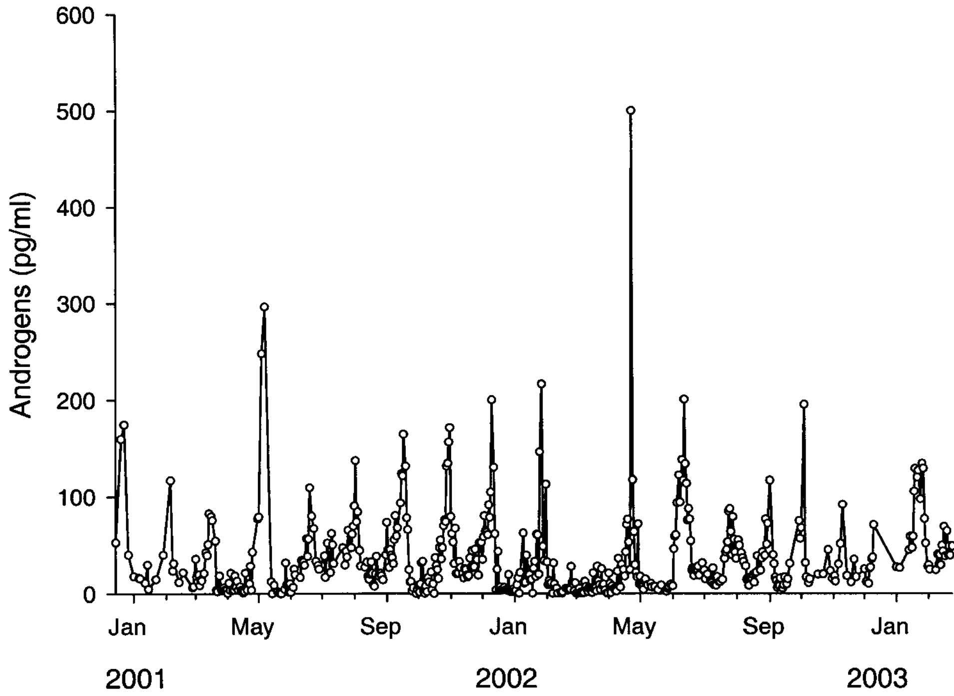

Fig. 3. Longitudinal profile of salivary androgens in a female Indian rhinoceros.

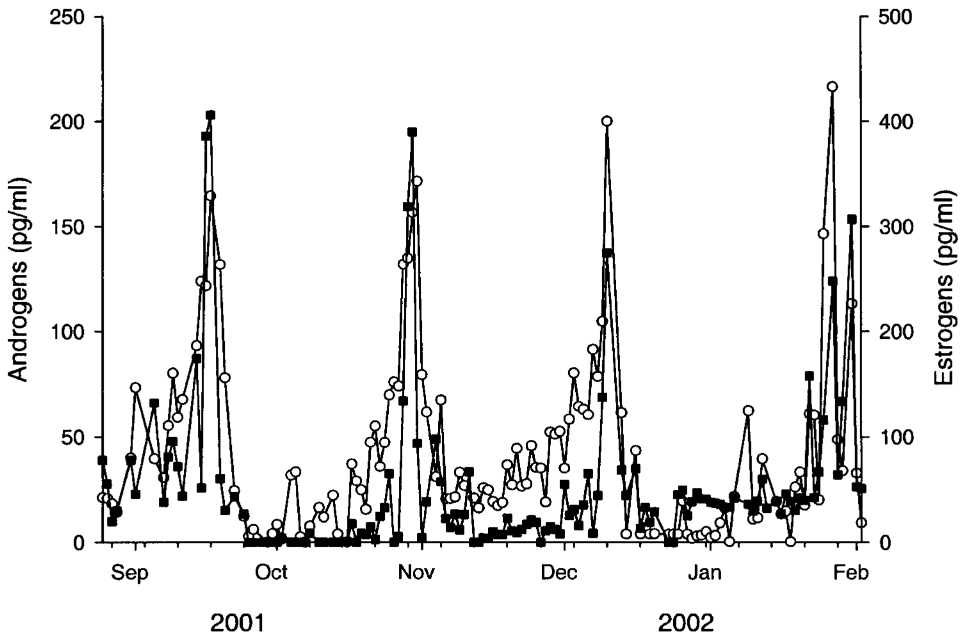

Fig. 4. Longitudinal profiles of salivary androgens (open circles) and estrogens (closed squares) in a female Indian rhinoceros.

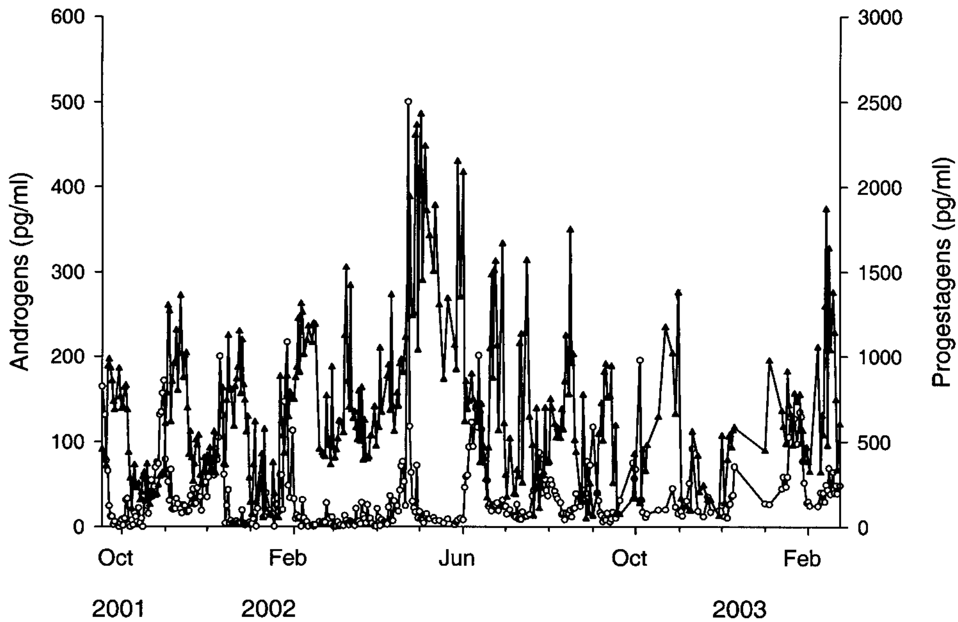

Fig. 5. Longitudinal profiles of salivary androgens (open circles) and progestins (closed triangles) in a female Indian rhinoceros.

hormones occurred simultaneously (Fig. 4). Peak androgen and estradiol concentrations varied between cycles (androgens: range = 79.7–500 pg/ml, median = 164.5 pg/ml; estradiol: range = 318.6–405.86 pg/ml, median = 387.71 pg/ml). Peak concentrations were clearly discernible for both hormones; however, the estrogen peaks were nearly twice the amplitude of the androgen peaks when compared to their respective baseline values.

Sustained increases in salivary progestin concentrations followed each androgen/estrogen peak. Based on this assessment, the mean duration of the luteal phase was 15.5 ± 1.5 days (range = 10–23 days, median = 16 days). There was one long cycle of 86 days in the spring of 2002. During this period, androgen concentrations remained at baseline, and progestins were variable but consistently elevated above baseline (Figs. 3 and 5). Following this cycle, the subsequent increases in salivary androgens and postovulatory progestins were the highest observed during the study period (Figs. 3 and 5). A similar aberrant cycle had not been observed the previous spring, although the androgen peak during the May 2001 cycle was the second-highest recorded during the study.

Peaks in salivary androgen metabolite excretion were associated with estrous behavior. Signs of estrus were detected for periods ranging from 2 to 16 days. Most frequently (7/11 cycles), behavioral estrus occurred for a period of time that was shorter than, and nested within the period of elevated androgen levels. These behaviors gradually intensified until they reached a peak, and then sharply decreased in a pattern similar to that of salivary androgens. By comparison, the expression of estrous behaviors was invariably longer than the period of elevated salivary estrogen concentrations. Vocalizations, urine-squirting, vaginal winking, and reduced

appetite were the most indicative signs of estrus, but they were not observed in every cycle. In general, behavioral observation was not a consistently reliable method for detecting impending estrus. Behavioral signs of estrus also varied among cycles, and, based on historical logs, some behaviors had changed over the years. Furthermore, during some cycles there was a total lack of estrous behaviors until the female was exposed to the male's stall. During the long cycle observed in the spring of 2002, the female showed no estrous signs around the time a new cycle was expected.

DISCUSSION

Previous studies on reproductive monitoring in female Indian rhinos were based on fecal and urinary analyses [Kassam and Lasley, 1981; Schwarzenberger et al., 2000]. In this study, it was not possible to collect uncontaminated urine or feces, so an alternative method had to be developed to assess reproductive cycles in the female. In an earlier study, Czekala and Callison [1996] used salivary hormone analyses to monitor pregnancy in black rhinos. Using a total estrogen RIA, they found a significant increase in salivary estrogens at the end of gestation, and HPLC analysis indicated significant immunoactivity associated with estradiol. Therefore, we tested several estrogen assays. Some were specific for estradiol-17β, and others were designed to measure total estrogens. None of the systems were effective for monitoring follicular activity in the saliva of this female. Instead, a testosterone EIA was found to produce displacement curves with serially diluted saliva extracts that were parallel to the standard curve, and cyclic profiles that matched observations of behavioral estrus. This assay was then used to track ovarian cycles for a 27-month period, and a total of 17 estrous cycles were documented. Later, a commercial EIA kit developed specifically for measuring estradiol in human saliva was found to be effective for assessing follicular cycles.

Similarly, we were unable to assess luteal function using a variety of EIA and RIA procedures. Although PdG is excreted in the urine of Indian rhinos [Kasman et al., 1986], we were unable to measure it in saliva using a specific PdG assay. Only a commercial EIA developed for human salivary progesterone was effective. It is not known why the commercial kits for estradiol and progesterone worked with Indian rhino saliva when the other assay systems did not. Estradiol and progesterone appear to be present in rhino saliva, and the HPLC analysis clearly identified progesterone as one of the immunoreactive peaks. Therefore, our inability to detect immunoactivity was probably not due to a lack of these hormones in the sample, or to problems with antibody specificity. Rather, it may have been due to assay or sample matrix effects. Even though the commercial kits were designed for unextracted saliva, the Indian rhino saliva had to be extracted and concentrated before we could obtain measurable results that were parallel to the respective standard curves. Thus, one should consider these factors when validating assay systems for new uses.

The ability of the testosterone EIA to monitor follicular activity in the Indian rhino is intriguing. Based on HPLC analysis, most of the androgen immunoactivity was due to androstenedione, a major follicular steroid produced in other species [Concannon and Verstegen, 1998; Drea et al., 1998]. Interestingly, Schwarzenberger et al. [2000] also found that androstenedione was a major fecal androgen metabolite in the Indian rhinoceros. In that study, fecal androgen concentrations increased

during the follicular phase in a pattern that was significantly correlated with the excretion pattern of fecal estrogens, and related to behavioral estrus [Schwarzenberger et al., 2000]. In contrast to the results of this study, the excretion patterns of fecal androgens and estrogens were of similar lengths [Schwarzenberger et al., 2000]. Nevertheless, studies of both saliva and feces have suggested that androgen metabolites are a good indicator of follicular activity in the Indian rhinoceros. Since androgen excretory patterns in other rhinoceros species have not yet been examined, it remains to be determined whether this approach has cross-species applications.

In general, follicular steroids in the Indian rhinoceros increase several days before they reach peak concentrations in conjunction with behavioral estrus [Kassam and Lasley, 1981; Schwarzenberger et al., 2000]. In the present study, salivary androgens increased gradually, beginning at the end of the luteal phase and continuing throughout the follicular phase, and then fell sharply after the peak. By contrast, the increase in salivary estrogens was of shorter duration, and the temporal rise and fall around the peak were of a similar duration. Both hormones peaked about the same day, however. Thus, the main difference between these two measures was that salivary androgens increased about 3 days earlier than the estrogens during the follicular phase. This difference may reflect changes in steroidogenic secretory activity between developing and mature follicles, since conversion of androgens to estradiol increases after dominant follicle selection [Van Voorhis, 1998]. Thus, both salivary androgen and estradiol analyses can be used to assess follicular activity in the Indian rhinoceros, although the lower cost of the testosterone EIA used in this study might make it a more practical tool for timing breeding introductions in the long term.

Analyses of salivary progestins (specifically 20α-OH-P) have been used successfully to diagnose pregnancy in black rhinoceroses [Czekala and Callison, 1996]. In the present study we found that salivary progestin analysis is also effective for monitoring nonpregnant luteal phases in the Indian rhinoceros, as concentrations increased after ovulation in a pattern similar to that reported for fecal and urinary progestin excretion [Schwarzenberger et al., 2000]. Based on the HPLC results, at least some progestin immunoactivity in Indian rhinoceros saliva was associated with progesterone; however, a number of other immunoactive peaks of greater and lesser polarity compared to progesterone were also observed. Presumably, this assay system would be useful for diagnosing pregnancy in the Indian rhinoceros as well.

Overall, the salivary steroid analyses resulted in findings similar to those obtained in earlier studies of female Indian rhinoceroses. In the current study, the cycle length was 47 days. Previous studies reported an overall cycle length of 43 [Kassam and Lasley, 1981; Schwarzenberger et al., 2000] or 48 [Kasman et al., 1986] days. Female Indian rhinos have been reported to be polyestrous and capable of cycling year round [Kasman et al., 1986; Kassam and Lasley, 1981; Schwarzenberger et al., 2000]. However, in the early spring of 2002, the NZP female exhibited a longer than average cycle (86 days). There were no behavioral signs of estrus during the period of low salivary androgens, when a new cycle would have been expected (around 45 days after the drop in androgens to baseline). Yet, the consistently elevated progestins suggest that ovulation did occur. A transient progestin drop observed mid-cycle suggests that an incomplete follicular cycle may have occurred. We also observed that the subsequent androgen rise and postovulatory progestin

concentrations were the highest measured during the study. Abnormal cycles have not been previously reported for the Indian rhinoceros [Kassam and Lasley, 1981; Kasman et al., 1986; Schwarzenberger et al., 2000], but other rhinoceros species have been shown to exhibit periods of acyclicity and wide variations in cycle length unrelated to season [Schwarzenberger et al., 1998; Patton et al., 1999; Brown et al., 2001; Graham et al., 2001].

In sum, this study reports the longest known use of salivary steroid analyses to monitor ovarian activity in a large mammalian wildlife species. Although eventually it was decided that the two NZP rhinos should not be bred (because of safety concerns for the female, and the potential for excessive aggression by the male), the techniques validated for assessing estrous cyclicity in this species could prove to be useful alternatives to noninvasive methods in cases in which it is impossible to collect uncontaminated urine or fecal samples.

CONCLUSIONS

1. Salivary androgen and estrogen metabolite levels are reliable indicators of follicular activity, and thus could be used as effective tools for timing breeding introductions.

2. Salivary progestin metabolite levels are reliable indicators of luteal function in the Indian rhinoceros, and may be useful for confirming ovulation and diagnosing pregnancy.

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