

Follicular, endocrine and behavioural dynamics of the Indian rhinoceros (*Rhinoceros unicornis*) oestrous cycle

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Abstract

Longitudinal ultrasound, behaviour and endocrine evaluations were conducted, over 14 to 18 months, in two young female Indian rhinoceroses (*Rhinoceros unicornis*) to characterize the oestrous cycle. Both females showed the same pattern of follicular development producing a large follicle (10–12 cm diameter) on one of the ovaries that persisted for 8.5 ± 4.68 days before spontaneously ovulating. Ovulation occurred in all eight cycles monitored in a 6- to 7-year-old female versus 10 out of 14 cycles monitored in a 5- to 6-year-old female. Ultrasound examinations confirmed follicular collapse 48 h following the onset of behavioural oestrus in ovulatory cycles, while anovulatory cycles were associated with the formation of a haemorrhagic follicle. The day of behavioural oestrus corresponded to peak urinary oestrogen conjugate concentrations for each cycle, but anovulatory cycles had lower concentrations on the day of behavioural oestrus compared with ovulatory cycles. A transient increase in urinary progesterone metabolite concentrations was detected 1 day prior to ovulation. Irregular urinary progesterone metabolite profiles followed anovulatory cycles, reflecting varying degrees of follicular luteinization. In an attempt to ensure that a cycle would result in ovulation in the 5- to 6-year-old female, a GnRH treatment was tested during two separate cycles. Administration of GnRH on the day of behavioural oestrus resulted in an increase in urinary luteinizing hormone concentrations 2 h following injection. Regardless, ovulation did not occur in response to treatment. This study provides the first ultrasound data on ovarian activity in the Indian rhinoceros and establishes normal physiologic and behavioural relationships during the oestrous cycle that may facilitate the breeding of this species in captivity.

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Introduction

The Indian rhinoceros (*Rhinoceros unicornis*) is the largest of the Asiatic rhinoceros species. Once ranging throughout most of Southeast Asia, wild populations of Indian rhinoceros are now found in two protected areas, southern Nepal and Assam, India. Wild population estimates for the Indian rhinoceros currently range from 2000 to 2100 animals (Foose & Van Strien 1997, Dinerstein 2003) with the captive population consisting of 138 animals distributed in 52 zoological institutions throughout North America, Europe, Asia and Australia (Hlavacek *et al.* 2002). Although captive breeding of the Indian rhinoceros has been rather successful in terms of the number of offspring produced, genetic representation is significantly skewed towards a few highly prolific founders (Zschokke *et al.* 1998, Hlavacek *et al.* 2002). In order to guarantee maximal genetic variation in subsequent generations of captive Indian rhinoceroses, breeding and successful reproduction must occur with regularity between specifically paired males and females.

Natural breeding attempts among captive Asian rhinoceros species frequently result in severe aggression between males and females (Khan *et al.* 1999, Roth & Brown 1999, Roth *et al.* 2001). Unlike the Sumatran rhinoceros (*Dicerorhinus sumatrensis*), the Indian rhinoceros typically exhibits overt signs of oestrous behaviour that traditionally have been used to time introductions for mating. Even so, many male Indian rhinoceroses have not sired offspring because they expressed significant aggression when introduced to a behaviourally oestrous female (Roth & Brown 1999). The ability to characterize ovarian, endocrine and behavioural dynamics associated with the periovulatory period in the Indian rhinoceros could greatly assist in timing introductions for mating and for developing assisted reproductive techniques, such as artificial insemination (AI), to help improve the genetic diversity of this species in captivity.

To optimize the likelihood of conception following natural breeding or AI, ovulation must occur near the time of insemination. Therefore, the timing of breeding or AI relies heavily on an understanding of female reproductive

physiology, yet reproductive data on most exotic animals are still very limited. However, recent advances in understanding female reproductive physiology in the Sumatran, African black (*Diceros bicornis*) and African white (*Ceratotherium simum*) rhinoceroses have expanded the knowledge base while revealing substantial variation in reproductive function among the rhinoceros species (Schwarzenberger *et al.* 1993, 1998, Berkeley *et al.* 1997, Radcliffe 1997, 2001, Roth *et al.* 2001, 2004, Patton *et al.* 1999, Roth & Brown 1999). Several of these studies utilized a combination of sera, faecal and/or urinary hormone analysis and rectal ultrasonography to delineate oestrous cycle dynamics, thereby producing information directly applicable to captive breeding efforts. For example, longitudinal analysis of serum progesterone concentrations and ultrasound imaging of ovarian and uterine tissue led to our understanding of Sumatran rhinoceros reproductive physiology and resulted in the first successful breeding of a captive Sumatran rhinoceros in 112 years (Roth *et al.* 2001, 2004). Similar information regarding the reproductive biology of the Indian rhinoceros is limited.

Both urinary and faecal hormone metabolite data matched to observations of behavioural oestrus have been used to monitor reproductive cyclicity in the Indian rhinoceros (Kassam & Lasley 1981, Kasman *et al.* 1986, Hodges & Green 1989, Schwarzenberger *et al.* 2000). These studies documented significant variability in the duration of the ovarian cycle, ranging from 39 to 64 days (Kassam & Lasley 1981, Kasman *et al.* 1986, Schwarzenberger *et al.* 2000). However, none of these studies incorporated ultrasonography in the methodologies employed. Therefore, detailed data on follicular dynamics, growth and ovulation do not exist. It was primarily through ultrasound monitoring that another Asiatic species, the Sumatran rhinoceros, was discovered to be an induced ovulator that exhibits a 21-day reproductive cycle when allowed to mate (Roth *et al.* 2001). In contrast, spontaneous ovulation has been documented by ultrasonography in both African black and African white rhinoceroses, with mean cycle lengths of 26 and 33 days respectively (Radcliffe *et al.* 1997, 2001).

The overall aim of this study was to characterize the Indian rhinoceros oestrous cycle and to establish the physiologic events associated with the periovulatory period that may help to improve captive breeding and AI success. Specific objectives were (1) to characterize ovarian activity by rectal ultrasonography; (2) to monitor behavioural oestrus in relation to ovulation; (3) to correlate urinary oestrogen conjugate (E_1C) and progesterone metabolite (PdG) concentrations excreted during the oestrous cycle to ovarian and behavioural dynamics; (4) to validate the use of a luteinizing hormone (LH) enzyme immunoassay (EIA) for measuring urinary LH; and (5) to determine if gonadotrophin releasing hormone (GnRH) administration during the periovulatory period would induce ovulation at a predictable time post-injection.

Materials and Methods

Animals

This research was conducted on two female Indian rhinoceroses maintained at the Cincinnati Zoo and Botanical Garden, Cincinnati, OH, USA. The females were maintained at the institution during separate time periods and are therefore presented as independent studies. Both females were captive born. The females had access to an outdoor exhibit (27 × 32 m) when weather allowed (temperature >7.2 °C) and had access to a pool (9 × 9 m) in their exhibit when the temperature was >15.6 °C. The exhibit for the female Indian rhinoceros was adjacent to the exhibit for the male Indian rhinoceros. The same male Indian rhinoceros (Studbook (SB) no. 147) was maintained at the institution throughout both studies. While housed inside, the female had access to 2 stalls (each 5 × 6 m) that were separated from the stall for the male Indian rhinoceros by a 6 m distance that contained the chute for conducting the ultrasound examinations and an empty pass-through used to shift the animals. The animals were exposed to artificial lighting in the indoor facility and, depending on the season, the facility was heated or cooled. Each day the female Indian rhinoceros was fed 1 bale of Timothy hay, 12 quarts of grain (ADF16; Mazuri, St Louis, MO, USA), 6 bananas, 6 carrots, 6 apples and 2 sweet potatoes. Each morning the female received 6 ml vitamin E supplement (Emcelle Tocopherol; 500 i.u./ml; Stuart Products Inc., Bedford, TX, USA) orally in a banana. In addition, the female was granted unlimited access to a mineral block and water. Fresh browse was offered opportunistically depending on the season.

Study no. 1

Indian rhinoceros (SB no. 189) 'Nikki' was born at the Toronto Zoo on 25 December 1991 and was 6 to 7 years of age during the study (8/5/98 to 8/7/99).

Study no. 2

Indian rhinoceros (SB no. 238) 'Chitwan' was born at the Smithsonian National Zoological Park on 18 September 1996 and was 5 to 6 years of age during the study (1/11/01 to 28/5/03). In January 2003, the artificial lights in the stalls housing the female were set to a fixed 14 h exposure regimen each day (0600 to 2000 h).

Rectal ultrasonography

The restraining device used during rectal ultrasonography was a steel constructed chute large enough to accommodate the entire animal. The animals entered and exited the chute through horizontally sliding concrete doors located at each end. The front door was closed as the female entered, with a hydraulic steel V-shaped designed back gate which closed after the female was in the chute. In addition, one entire side of the chute could move laterally

to decrease the amount of lateral movement the female could achieve during the examination. The female was in the chute for 10–15 min for each examination. She was provided with feed as part of the conditioning process. An Aloka 500 machine (Aloka, Wallingford, CT, USA) connected to a Hi-8 video or CD recorder and a 5 MHz linear array probe were used for all examinations. A Sony thermal printer (Aloka) was used to print the images from the Hi-8 video recorder, while Terapin Video Editing Software (Total Machine Vet Services, Orient, OH, USA) was used to capture images as jpeg files from the CD. Because the left ovary was anatomically located beyond the reach of the palpator, use of an extension attached to the probe was required to view the entire left uterine horn and ovary. The start of ultrasound examinations denoted day 1 of each study. During the examination, the bladder, cervix, uterine body, uterine horns and both ovaries were examined. Ovarian follicles >1 cm in diameter were measured and the presence of luteal tissue was recorded. Each female was examined 3 to 7 times each week. Day 0 of an oestrous cycle was defined as the day ovulation was confirmed by ultrasonographic visualization of follicular collapse followed by the formation of a luteal structure.

Study no. 1

Ultrasound examinations were conducted on female no. 189 from day 1 to day 438 (8/5/98 to 8/7/99) with the exception of days 193 to 257 (30/11/98 to 2/2/99).

Study no. 2

Ultrasound examinations were conducted on female no. 238 from day 1 to day 575 (1/11/01 to 28/5/03). When follicle size reached >10 cm in diameter, daily examinations were performed. With the onset of behavioural oestrus, ultrasound examinations were performed at 12, 24, 36, 48 and 72 h post-oestrus to correlate timing of ovulation in relation to behavioural oestrus. During behavioural oestrus, the female did not show interest in feed while in the chute, so fresh male Indian rhinoceros faeces was used to hold her attention while the ultrasound procedure was performed.

Collection of urine samples

Keeper staff obtained fresh urine samples by aspiration off the stall floor or collecting directly into a storage vial when the female urinated with her body oriented to facilitate mid-stream collection.

Study no. 1

Fresh urine samples were opportunistically collected from day -14 to day 435. All urine samples were frozen (-20°C) immediately following collection in 5 ml round bottom polypropylene tubes (Corning Incorporated, Corning, NY, USA) and subsequently assayed for E₁C and PdG concentrations.

Study no. 2

Fresh urine samples were collected opportunistically from day -87 to day 575. An aliquot of urine (1 ml) was kept at refrigerator temperature in a 1.5 ml Eppendorf tube for subsequent LH analysis, while the remaining volume of urine was frozen (-20 °C) in a 5 ml polypropylene round bottom tube and later assayed for E₁C and PdG concentrations.

Enzyme immunoassays

Studies 1 and 2

Concentrations of urinary E₁C and PdG were measured using EIA techniques described by Munro *et al.* (1991). Polyclonal antibodies R522 and R13904 (supplied by C Munro, University of California, Davis, CA, USA) were utilized in the E₁C and PdG EIAs respectively. Standard concentrations for both assays were 3.9 to 250 pg well⁻¹. The E₁C and PdG EIAs were validated for Indian rhinoceros urine by demonstrating parallelism between standard curves and serial dilutions of pooled urine (neat to 1:640). Frozen-thawed urine samples were run at a 1:80 dilution in EIA buffer for both assays. Interassay coefficients of variation for the E₁C assay were 19% and 20% for internal controls at 38% and 83% binding respectively. Interassay coefficients of variation for the PdG assay were 19% and 13% for internal controls at 42% and 86% binding respectively. Creatinine concentrations were measured using a commercially available kit (no. 558-A; Sigma Diagnostics, St Louis, MO, USA). Urine samples were diluted 1:10 in double-distilled water (H₂O) for the creatinine assay. Urinary concentrations of steroid hormones were indexed by creatinine concentration in the urine and expressed as ng mg⁻¹ creatinine (Cr).

Study no. 2

Concentrations of LH in Indian rhinoceros urine samples were measured using an EIA originally developed to detect LH in elephant sera (Graham *et al.* 2002). Microtiter plates (96-well Maxisorp Nunc Immunoplates; Nalge Nunc International, Rochester, NY, USA) were coated with 50 µl monoclonal mouse anti-bovine LHβ antibody (Quidel clone no. 518-B7; supplied by J Roser, University of California, Davis, CA, USA) at a 1:15 000 dilution in coating buffer, covered with an acetate plate sealer and placed at room temperature for a minimum of 12 h. Following incubation, all wells of the microtiter plate were washed 5 times with wash solution and blotted dry. LH assay buffer was added to the plate (50 µl well⁻¹) and equilibrated at room temperature for 30 min. Following equilibration, 50 µl NIH-bovine LH standards (A F Parlow; NIDDK, National Hormone and Pituitary Program, Torrance, CA, USA) and Indian rhinoceros urine samples were added to the plate in duplicate. Plates were sealed and incubated overnight at room temperature. After overnight incubation, 50 µl biotinylated NIH-ovine LH

(A F Parlow, NIDDK National Hormone and Pituitary Program) were added to each well at a 1:8000 dilution in LH assay buffer and allowed to compete for 3 h at room temperature. Plates were washed 5 times, blotted dry and 100 μ l streptavidin-peroxidase conjugate (Roche Molecular Biochemicals, Indianapolis, IN, USA) at a 1:16 000 dilution in LH assay buffer were added to each well. Plates were again washed 5 times, blotted dry and 200 μ l LH substrate solution (24 ml LH substrate buffer, 100 μ l 0.1752 M H₂O₂ and 0.5 ml 0.016 M tetramethylbenzidine in dimethylsulphoxide) were added to all wells. Plates were allowed to develop for 1 h at room temperature before stopping the reaction with 50 μ l well⁻¹ of 4 M sulphuric acid.

Standard concentrations were 3.9 to 250 pg well⁻¹. A dose-response analysis of pooled unfrozen urine samples collected from female no. 238 following GnRH treatment no. 1 was used to demonstrate parallelism to the standard curve. Interassay coefficients of variation for the LH assay were 18% for internal controls at 20% and 88% binding. For the LH assay, unfrozen urine samples were run undiluted in duplicate, indexed for Cr and expressed as pg LH mg⁻¹ Cr. The LH assay was also validated using Sumatran rhinoceros serum by demonstrating parallelism to the standard curve of serially diluted Sumatran rhinoceros serum samples (neat to 1:16) previously determined by radioimmunoassay to contain high and low concentrations of LH.

Daily observations

Study no. 2

Indian rhinoceros keeper staff filled out daily observation sheets detailing specific information on the female and the male. On the sheet, keeper staff documented the amount of feed given to and eaten by the female, time out and in from exhibit, and behavioural observations. The female was given a small amount of fresh male Indian rhinoceros faecal sample on a daily basis to monitor her behavioural response. In addition, the days in which the female excreted vaginal discharge were noted. Daily rectal temperatures were taken on the female during the early morning of days 368 to 439 of the study (24/10/02 to 3/1/03). Daily information was also collected on the male Indian rhinoceros. These observations included vocalization, change in appetite and degree of ease or difficulty met by keeper staff in getting the male out on exhibit or moving him from one stall to another.

GnRH treatment trials

Study no. 2

A GnRH injection was tested during two separate oestrous cycles of the female Indian rhinoceros. A single injection of GnRH (Cystorelin; 50 μ g ml⁻¹ (w/v) GnRH diacetate tetrahydrate, 10 ml i.m.; CEVA Laboratories, Overland Park, KS, USA) was administered via remote dart delivery

on the day of behavioural oestrus, peak urinary E₁C concentrations and ultrasound evidence of a preovulatory follicle (diameter >10 cm). In the first trial, an ultrasound was conducted at 4 h post-injection and then every 6 h until 76 h post-injection. In the second trial, an ultrasound was conducted at 4 h post-injection and then every 6 h until 48 h post-injection. Throughout both trials, aliquots of all urine samples excreted by the female were collected, stored at 4°C and subsequently assayed for LH concentrations.

Statistical analyses

The Sigma Plot/Sigma Stat software program (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Pearson product moment correlation was used to compare the serial dilutions of urine to the standard curve for all assays. Standard descriptive statistics were used to summarize results and statistical significance was tested using Student's *t*-test. Oestrous cycle length was calculated in two ways: based on the date ovulation was detected by rectal ultrasonography and based on alignment of peak urinary oestrogen conjugate concentrations. The lengths of the follicular phase and luteal phase of all oestrous cycles were calculated. The follicular phase started on the day urinary E₁C concentrations rose above baseline and ended on the day ovulation was detected by rectal ultrasonography. The luteal phase started on the day of ovulation and ended on the day urinary PdG concentrations returned to baseline. Means \pm standard deviation (s.d.) are reported. The hormone data for the three oestrous cycle patterns exhibited by female no. 238 were aligned according to the day of behavioural oestrus. Values were arranged in periods of 15 days before and 30 days after behavioural oestrus and are graphically presented as means \pm standard error of the mean (s.e.m.). For oestrous cycles associated with two separate days of behavioural oestrus, data were aligned according to the second behavioural oestrus. The three oestrous cycle patterns were compared using one-way analysis of variance (ANOVA). If a statistically significant difference resulted, pairwise multiple comparison procedures were employed using the Student-Newman-Keuls method if equal variance passed and the Kruskal-Wallis one-way ANOVA on ranks if equal variance failed. Regression analysis was employed to characterize the follicular growth pattern for each of the three oestrous cycle patterns exhibited by female no. 238 in Study no. 2.

Results

Studies 1 and 2

Oestrous cycle dynamics

The ultrasonographic and endocrine dynamics obtained from individual oestrous cycles (*n* = 22) in the two female Indian rhinoceroses studied are presented in Table 1. In both females, a dominant follicle grew on one of the

Table 1 Comparison of ultrasonographic and endocrine characteristics of the oestrous cycles of two female Indian rhinoceroses. Data are expressed in means \pm S.D.

Female Studbook no. 'Name'	Age during study (years)	Number of oestrous cycles	Ovulatory cycles observed	Anovulatory cycles observed	Mean cycle length (days) ^a	Peak E ₁ C concentrations during follicular phase (ng mg ⁻¹ Cr)	Peak PdG concentrations during luteal phase (ng mg ⁻¹ Cr)	Follicular phase (days) ^b	Luteal phase (days) ^c	Preovulatory follicle diameter (cm)
no. 189 'Nikki'	6-7	8	8	0	43.8 \pm 5.93	775.0 \pm 492.6	1500.8 \pm 765.7	13.6 \pm 4.96	17.0 \pm 4.9	10-12
no. 238 'Chitwan'	5-6	14	10	4	43.0 \pm 7.53	1654.6 \pm 868	1319.3 \pm 1215.9	13.5 \pm 3.21	17.0 \pm 3.38	10-12
Combined females	5-7	22	18	4	43.3 \pm 6.78	1373.8 \pm 958	1397.7 \pm 1015	13.6 \pm 3.84	17.0 \pm 4.26	10-12
Statistical analyses	—	—	—	—	<i>P</i> = 0.842	<i>P</i> = 0.004*	<i>P</i> = 0.755	<i>P</i> = 0.989	<i>P</i> = 0.724	—

P is the degree of significance, with *P* < 0.05 indicative of a significant difference (*).

^aBased on interval between confirmed ovulations; ^bbased on interval between urinary E₁C concentrations measuring above baseline to ovulation; ^cbased on interval from ovulation to day urinary PdG concentrations returned to baseline.

ovaries and reached a maximal diameter of 10–12 cm prior to ovulation (Fig. 1a). It was not until the follicle reached >9 cm in diameter in female no. 189, and >8 cm in diameter in female no. 238, that E₁C concentrations rose above baseline. Baseline E₁C concentrations for female no. 189 and no. 238 were 16.0 \pm 21.0 and 48.1 \pm 37.7 ng mg⁻¹ Cr respectively. Urine samples with E₁C concentrations >58.0 ng mg⁻¹ Cr for female no. 189 and >123 ng mg⁻¹ Cr for female no. 238 were above baseline and marked the beginning of the follicular phase. While there was no statistical difference in the length of the follicular phase, the mean peak E₁C concentrations measured during the follicular phase differed (*P* < 0.05) between females, with female no. 189 producing lower peak E₁C concentrations (Table 1). Ovulation was verified by rectal ultrasonography in all oestrous cycles monitored in female no. 189 and in 71% of the oestrous cycles monitored in female no. 238. Ovulation was characterized by complete follicular collapse (Fig. 1b) followed 1 day later by the formation of a corpus haemorrhagicum (Fig. 1c). The length of the oestrous cycle, based on one complete interovulatory interval, did not differ between females. Similarly, based solely on the interval between peak E₁C concentrations, mean cycle length in the two females was 43.0 \pm 6.0 days. The luteal phase in the two females did not differ in length or in the peak urinary PdG concentrations achieved. Fluid was often observed within the lumen of the uterine horns 17.0 \pm 2.35 days following ovulation (Fig. 1d), which corresponded to the time of precipitous decline in urinary PdG concentrations.

Study no. 2

Female and male Indian rhinoceros behaviour

Behavioural oestrus was identified by specific changes in the female's behaviour that included a marked decrease in appetite, an increase in activity level, vocalization and frequent urine spraying associated with vulva winking. In all cycles, the male Indian rhinoceros showed behavioural changes that preceded behavioural oestrus of the female by 2–3 days. These changes included inappetence overnight, an increase in vocalization (whistling and roaring) when both indoors and outdoors, and extreme resistance to leaving the barn where the female was held.

Ovulatory oestrous cycle dynamics

Of the 14 oestrous cycles monitored by rectal ultrasonography in female no. 238, a total of ten cycles were associated with successful ovulation (Fig. 2). The growth of the preovulatory follicle and subsequent ovulation occurred with equal frequency from both ovaries. Based on the interval between follicular collapses, ovulatory oestrous cycles ranged in length from 36 to 61 days and were characterized by two distinct behavioural patterns. The female either exhibited 1 day of behavioural oestrus with ovulation occurring 48 h post oestrus (*n* = 6 cycles), or

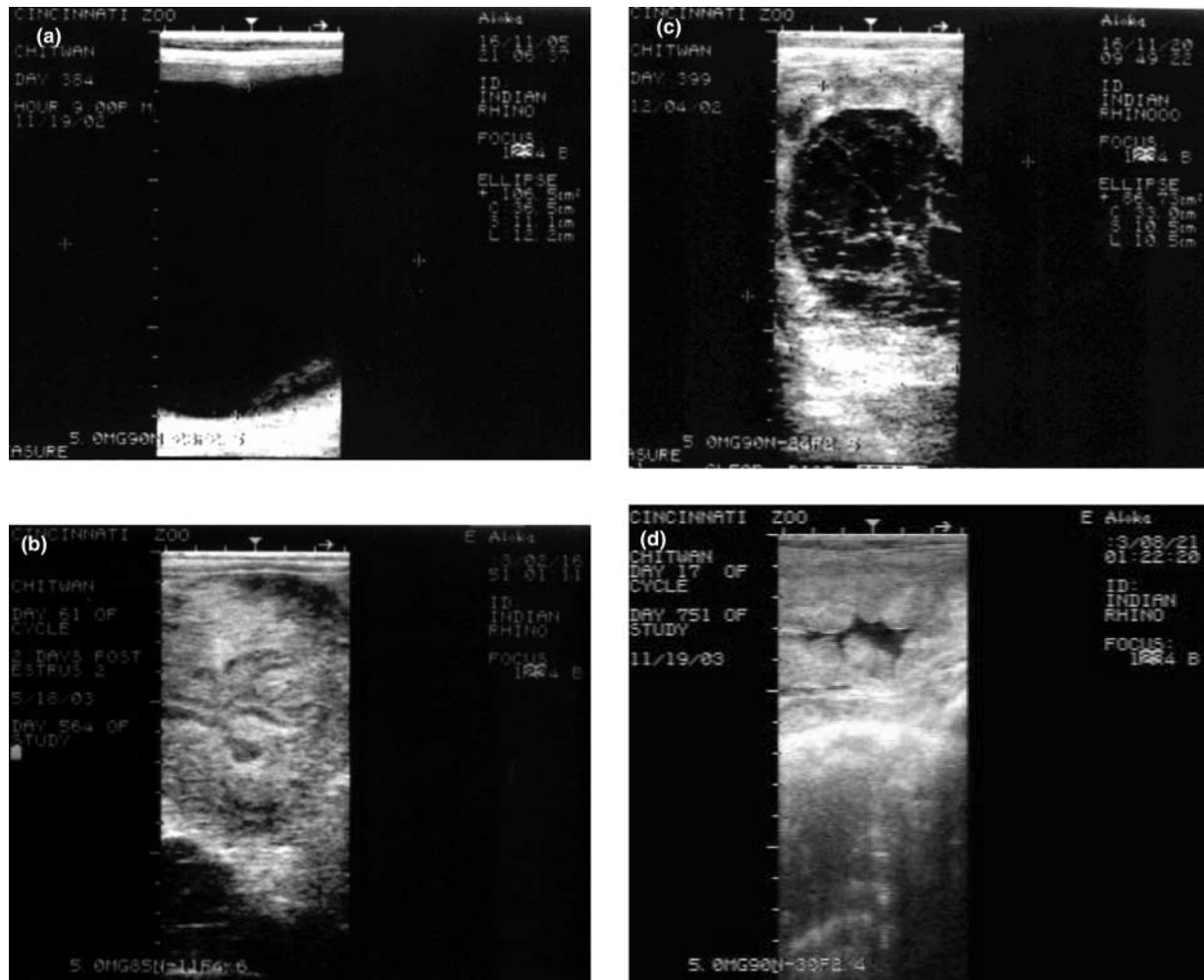


Figure 1 Ultrasonographic images obtained during the oestrous cycles of female 238. (a) Preovulatory follicle associated with an oestrous cycle in which 1 day of behavioural oestrus was exhibited. This ultrasonographic image of the preovulatory follicle was taken 39 h post oestrus and only 9 h prior to ovulation. (b) Ultrasonographic image of follicular collapse that confirmed ovulation 48 h post-oestrus. (c) A day 15 corpus haemorrhagicum that had organizing criss-crossing fibrin strands. (d) Ultrasonographic image of fluid within the lumen of the uterine horn day 17 post ovulation.

she exhibited 1 day of behavioural oestrus, followed by 1–3 days of normal behaviour, then a second day of behavioural oestrus with ovulation occurring 48 h later ($n = 4$ cycles). The two behavioural patterns were associated with cycles of different length or interovulatory period ($P < 0.05$), with the oestrous cycle averaging 37.8 ± 1.95 days for the former and 49.0 ± 8.58 days for the latter (Fig. 2). No differences ($P > 0.05$) were observed in the length of the follicular or luteal phases between the two patterns. The follicular phase was 13.17 ± 3.43 days for oestrous cycles with one behavioural oestrus and 14.0 ± 3.74 days for those with 2 separate days of behavioural oestrus. The mean concentrations of E_1C during the follicular phase were similar ($P > 0.05$) between ovulatory cycles with one or 2 separate days of behavioural oestrus ($1475.8 \pm 475 \text{ ng mg}^{-1} \text{ Cr}$ and $2567.8 \pm 1219.5 \text{ ng mg}^{-1} \text{ Cr}$ respectively) (Fig. 3a,b). However, urinary E_1C concentrations on the day of

behavioural oestrus were higher ($P < 0.05$) in oestrous cycles with 2 separate days of behavioural oestrus versus cycles with one day of behavioural oestrus. In those oestrous cycles in which 2 separate days of behavioural oestrus were exhibited, urinary E_1C remained at peak concentrations throughout the day(s) between oestrus exhibitions (Fig. 3b). With both behavioural patterns, E_1C concentrations reached baseline 3.9 ± 1.37 days following the oestrus that preceded ovulation (Fig. 3a,b). The preovulatory follicle was always present at 36 h post-oestrus but had collapsed at 48 h. Within 24 h following confirmation of follicular collapse, the central clot of the corpus haemorrhagicum was detectable by ultrasound. A transient rise in PdG concentrations ($> 200 \text{ ng mg}^{-1} \text{ Cr}$) was measured in urine collected 1 day following oestrus, which corresponded to the day prior to ovulation (Fig. 3a,b). The sustained rise in urinary PdG concentrations indicating the initiation of luteal tissue formation and

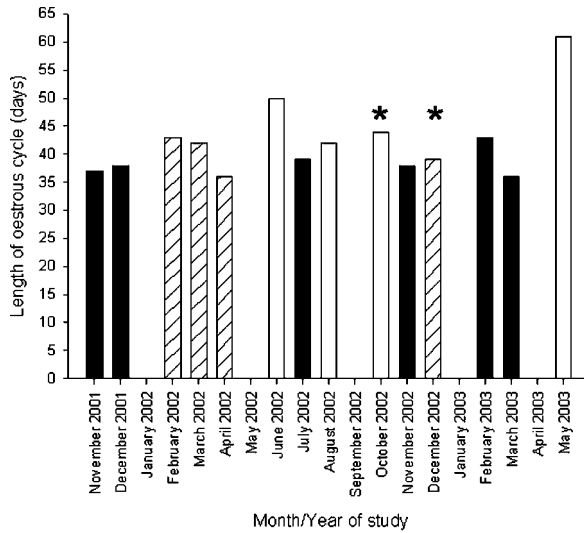


Figure 2 Longitudinal profile of the occurrence and length of the three oestrous cycle patterns exhibited by female no. 238 during Study no. 2. Length of the oestrous cycle was based on the interval between ultrasonographic confirmation of ovulation for ovulatory cycles, and the day of behavioural oestrus for anovulatory cycles. The study encompassed 14 oestrous cycles. A total of 10 ovulatory cycles occurred that were associated with either 1 (solid bars, $n = 6$) or 2 (open bars, $n = 4$) days of oestrus behaviour. In addition, four anovulatory cycles (hatched bars) were documented that were associated with 1 day of behavioural oestrus. GnRH treatment (*) was tested in two separate oestrous cycles.

function occurred 5 days following ultrasonographic confirmation of ovulation (Fig. 3a,b). Neither the length of the luteal phase (17.0 ± 3.38) nor peak PdG concentrations ($1319.3 \pm 1215.9 \text{ ng mg}^{-1} \text{ Cr}$) differed ($P > 0.05$) between cycles associated with the two different behavioural patterns (Fig. 3a,b).

Anovulatory oestrous cycle dynamics

A total of four anovulatory oestrous cycles were diagnosed in female no. 238 by rectal ultrasonography (Table 1). Anovulatory oestrous cycles were associated with just 1 day of behavioural oestrus followed by the formation of a haemorrhagic follicle. The female grew a follicle on one of the ovaries that reached a mean diameter of $11.1 \pm 0.97 \text{ cm}$ on the day of behavioural oestrus (Fig. 4a). Peak E_1C concentrations excreted during these cycles measured $1155.0 \pm 306.4 \text{ ng mg}^{-1} \text{ Cr}$ and became baseline 5.3 \pm 3.3 days post-oestrus (Fig. 3c). Urinary E_1C concentrations were lower ($P < 0.05$) on the day of behavioural oestrus in anovulatory compared with ovulatory oestrous cycles (Fig. 3a,b,c). Because no follicular collapse was associated with anovulatory cycles, day 0 of these cycles was determined by the day of behavioural oestrus. Anovulatory oestrous cycles had a mean cycle length of 40 ± 3.16 days (Fig. 2). Anovulatory follicles occurred three separate times on the right ovary and once on the left ovary. The four anovulatory oestrous cycles occurred from January to April 2002 and again in

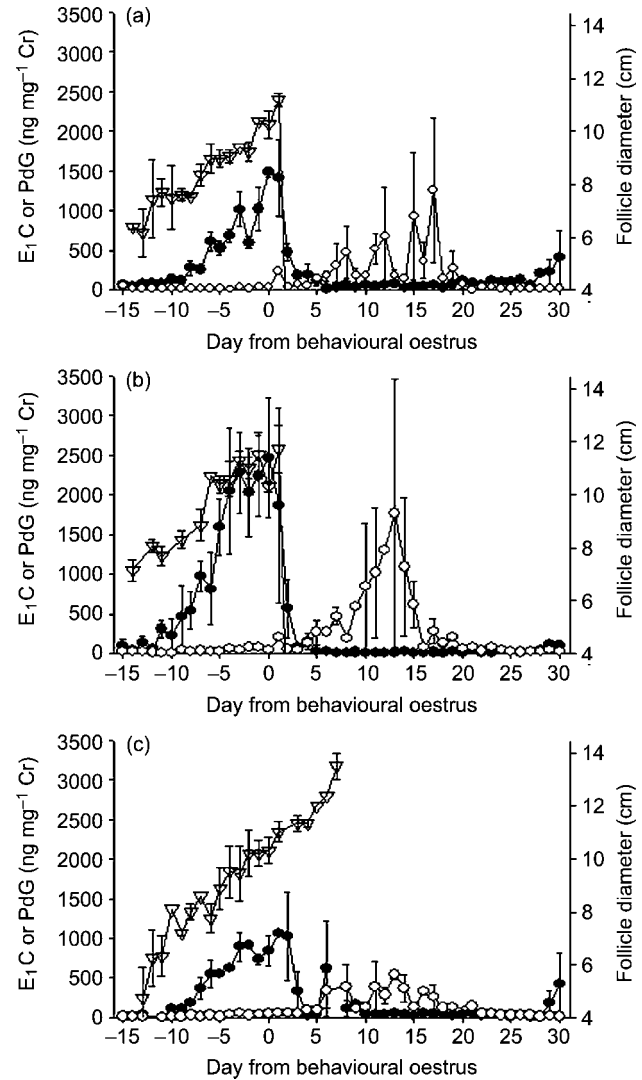


Figure 3 Composite profiles of the follicular growth pattern of the dominant follicle (∇) and urinary oestrogen conjugate (E_1C , \bullet) and progesterone metabolite (PdG, \circ) concentrations (mean \pm S.E.M.) excreted throughout the oestrous cycle for the three patterns exhibited by female no. 238. Data are aligned from the day of behavioural oestrus. (a) Profile for ovulatory oestrous cycles associated with 1 day of behavioural oestrus ($n = 6$ cycles). (b) Profile for ovulatory oestrous cycles associated with 2 separate days of behavioural oestrus ($n = 4$ cycles). For those ovulatory oestrous cycles associated with 2 separate days of behavioural oestrus, day 0 corresponds to the second behavioural oestrus. (c) Profile for anovulatory oestrous cycles associated with the formation of a haemorrhagic follicle ($n = 4$ cycles).

December 2002 (Fig. 2). Haemorrhagic follicles were diagnosed based on persistent maximal follicle diameter following behavioural oestrus, in some cases exceeding the preovulatory diameter (range 12.9–14.9 cm; Fig. 3c), no occurrence of follicular collapse, and the presence of uncoagulated blood swirling within the follicle upon ballotement (Ginther 1995). These follicles progressed from containing slightly echogenic follicular fluid to eventual complete infiltration of the follicular lumen (Fig. 4b). As

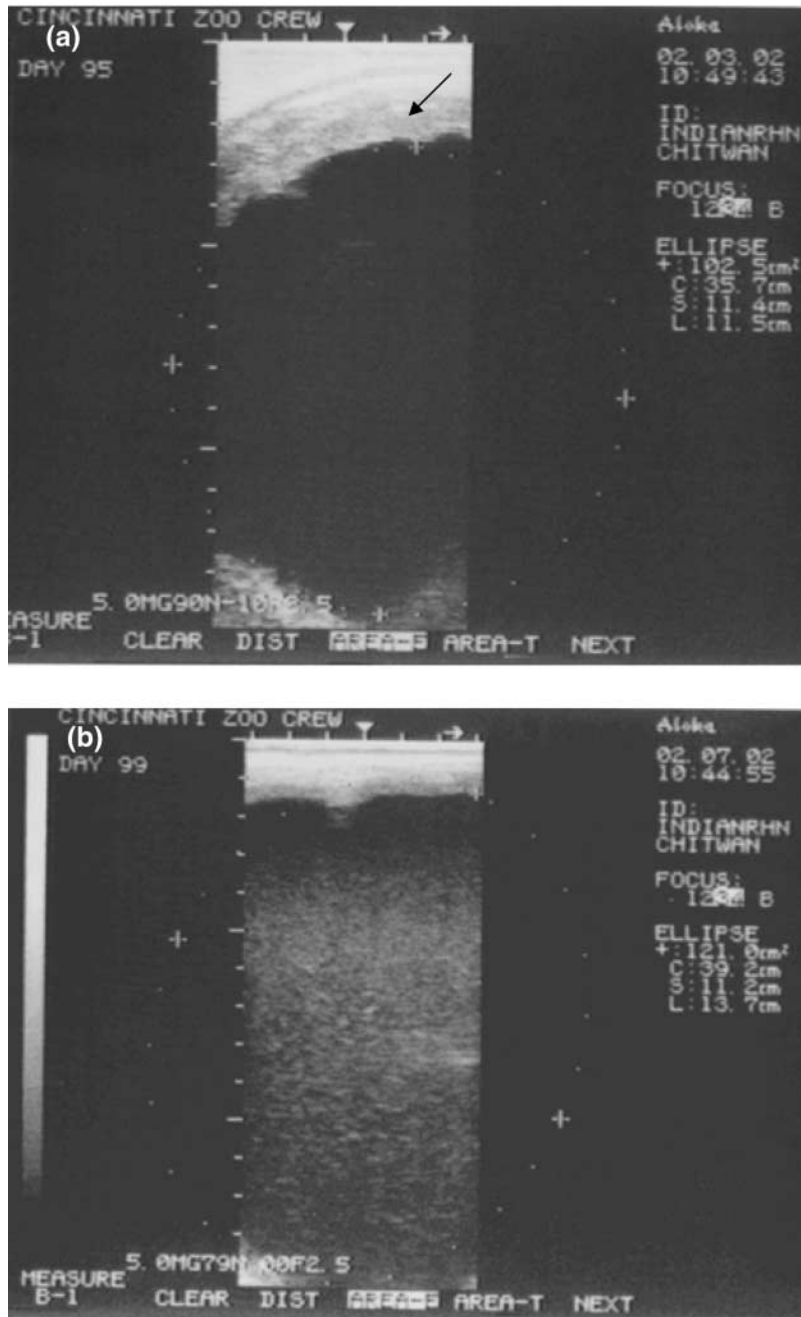


Figure 4 Ultrasonographic images obtained during the oestrous cycles of female no. 238. (a) Ultrasonographic image of an anovulatory follicle on the day of behavioural oestrus. Note the similar size to the preovulatory follicle (Fig. 1a) and the thickened follicular wall (arrow) indicative of estrogen producing cells. (b) Ultrasonographic image of an anovulatory haemorrhagic follicle 4 days following behavioural oestrus. Note the echogenicity of the follicular fluid and the thinning follicular wall. Upon ballottement, the fluid within the follicle swirled. (c) A day 8 anovulatory haemorrhagic follicle. Note the distinct pockets observed within the structure. While the upper pocket (UP) had distinct gel-like bands, fluid was observed to swirl in the lower pocket (LP) upon ballottement. (d) Ultrasonographic image of a large fluid-filled structure diagnosed as a follicular cyst. Note the absence of a thickened follicular wall.

the haemorrhagic follicles slowly filled, they formed distinct pockets containing fluid and fibrin bands (Fig. 4c). These follicles gradually regressed and were no longer visible by ultrasound at 30 days. All anovulatory cycles were associated with some PdG production post oestrus, but peak concentrations were markedly lower ($607.3 \pm 305.9 \text{ ng mg}^{-1} \text{ Cr}$) than those measured for oestrous cycles associated with successful ovulation (Fig. 3c). No transient rise in PdG concentrations was measured in urine collected 1 day following oestrus in any of the four anovulatory oestrous cycles observed (Fig. 3c).

Follicular growth pattern

In all cycles monitored, several small follicles (1–5 cm) often developed early in the cycle but only one grew further to become a dominant preovulatory follicle. Ovulatory oestrous cycles with one occurrence of behavioural oestrus were associated with emergence of the dominant follicle earlier in the cycle compared with the other oestrous cycle patterns. Growth of the dominant follicle during ovulatory oestrous cycles did not differ between the two behavioural patterns and occurred at a rate of $0.39 \pm 0.04 \text{ cm day}^{-1}$ once the follicle reached

6 cm in diameter until maximal diameter was achieved. However, the length of time maximal follicle diameter was maintained prior to ovulation did differ between the two behavioural patterns, with the dominant follicle retained 3.2 ± 1.79 days (Fig. 3a) prior to ovulation in cycles with one behavioural oestrus versus 7.75 ± 1.5 days (Fig. 3b) in cycles with 2 separate days of behavioural oestrus. Growth of the dominant follicle for anovulatory cycles occurred at a rate of 0.32 ± 0.04 cm day⁻¹ once the follicle reached 6 cm in diameter until a 10 cm diameter was achieved. The anovulatory follicles then grew at 0.5 ± 0.06 cm day⁻¹ until infiltration was complete (Fig. 3c).

Follicular cyst

There was one cycle in which a follicular cyst formed that resulted in the extension of an ovulatory oestrous cycle to 61 days (Fig. 2; Fig. 4d). The female quickly grew a large follicle on the right ovary that reached a 9 cm diameter by day 24 of the cycle and reached a maximal diameter of 11 cm by day 45 of the cycle. Throughout the growth of the follicle, it appeared thin walled, and urinary E₁C and PdG concentrations remained at baseline. From day 38 to day 61 of the oestrous cycle, the left ovary developed a follicle that grew to become the preovulatory follicle. The follicular cyst gradually declined from 7 cm on day 48 to 4 cm on day 54 of the oestrous cycle.

Vaginal discharge

Vaginal discharge for ovulatory cycles associated with one behavioural oestrus occurred at a mean of 3.5 days post oestrus and lasted 7 days post ovulation. Vaginal discharge was noted prior to ovulation in those ovulatory oestrous cycles with two exhibitions of behavioural oestrus. The pattern of discharge for these cycles was 1 day following the second oestrus through 7 days post ovulation. In the case of anovulatory cycles, only 1 day of vaginal discharge was noted at 5 days post oestrus.

Rectal temperatures throughout the oestrous cycle

During two separate oestrous cycles, rectal temperatures were taken daily. Both cycles were associated with just one day of behavioural oestrus. In November 2002, the female completed a successful ovulatory cycle and in December 2002 an anovulatory cycle occurred. Mean rectal temperature throughout these two cycles was 37.1°C. The day of behavioural oestrus for each of the two cycles was associated with a rise in rectal temperature to 38.2°C and 38.0°C respectively ($P < 0.05$).

LH EIA validation

A parallel dose-response was observed between serially diluted (neat to 1:32) female Indian rhinoceros urine samples and bovine LH standards throughout the range of the curve. The correlation coefficient between the standard and pooled urine was 0.992 ($P < 0.05$).

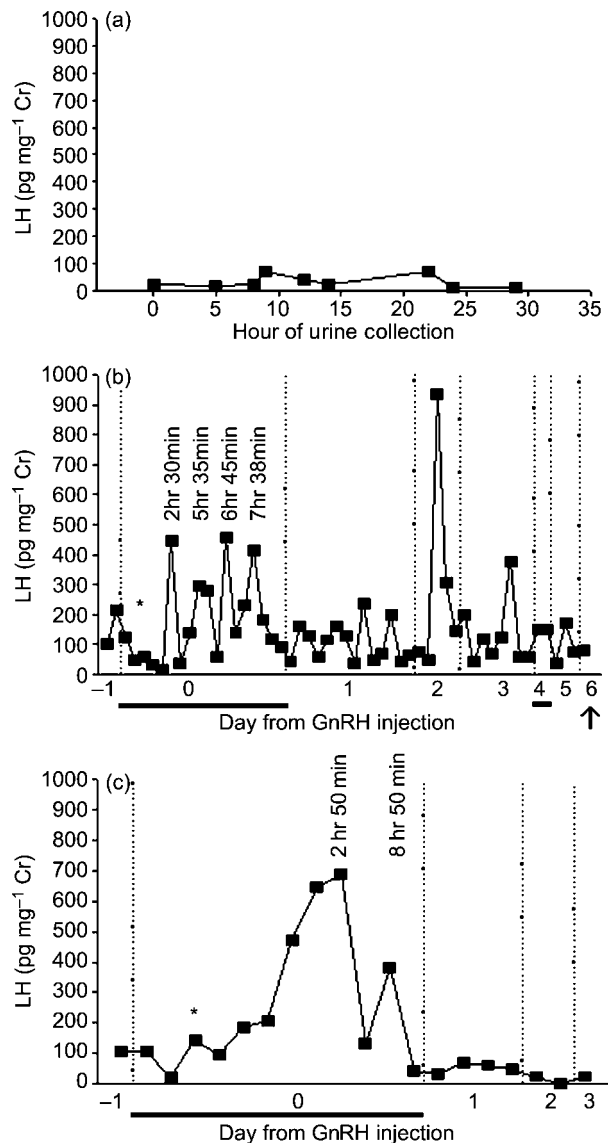


Figure 5 Urinary LH profiles excreted by female no. 238. (a) Baseline LH concentrations excreted during a 24 h period of day 29 and day 30 of an oestrous cycle. (b) Pattern of LH excretion following a GnRH injection (*) administered during the first oestrus of an ovulatory oestrous cycle associated with two separate days of behavioural oestrus. (c) Pattern of LH excretion following a GnRH injection (*) administered during oestrus of an anovulatory oestrous cycle. Days on which behavioural oestrus were observed are noted by thick black lines below the x-axis and the day ovulation was detected by rectal ultrasonography is denoted by an arrow.

Baseline urinary LH

Mean urinary LH concentration excreted during a 24 h time period on days 29 and 30 of an oestrous cycle was 34.7 ± 8.52 pg mg⁻¹ Cr (Fig. 5a).

GnRH treatment Trial no. 1

On 7 October 2002 (day 38 of the oestrous cycle), the female exhibited behavioural signs of oestrus. An

ultrasound conducted on the morning of oestrus detected a follicle measuring 11.8 cm in diameter and the E_1C concentration of an early morning urine sample was $1213 \text{ ng mg}^{-1} \text{ Cr}$. At 1157 h on 7 October 2002 $500 \mu\text{g GnRH}$ were administered to the female. Retrospective LH EIA analysis of urine samples collected from Trial no. 1 indicated that four LH surges occurred on the day of oestrus following the GnRH injection (Fig. 5b). The first LH surge was measured in urine 2 h and 30 min following GnRH injection with the fourth surge measured at 7 h and 38 min. On day 2 and 3 post-GnRH injection, LH surges that measured 936 and $374 \text{ pg mg}^{-1} \text{ Cr}$ respectively were detected (Fig. 5b). The female exhibited a second behavioural oestrus on the fourth day after GnRH injection. Ovulation was detected by rectal ultrasonography on day 6 post-GnRH injection, 48 h after the second behavioural oestrus. Mean E_1C concentrations measured $>1000 \text{ ng mg}^{-1} \text{ Cr}$ throughout the trial until the day of ovulation, when the mean concentration measured $407 \text{ ng mg}^{-1} \text{ Cr}$. Mean PdG concentrations measured $39.7 \pm 33.0 \text{ ng mg}^{-1} \text{ Cr}$ throughout the trial until day 5 post-GnRH injection when the mean concentration measured $203 \text{ ng mg}^{-1} \text{ Cr}$. Day 5 post-GnRH injection corresponded to 1 day after the second oestrus, and 1 day prior to ovulation. Throughout the trial, LH appeared pulsatile and was consistently above baseline.

GnRH treatment Trial no. 2

On 29 December 2002 (day 39 of the oestrous cycle), the female exhibited behavioural signs of oestrus. An ultrasound conducted on the morning of oestrus detected a follicle measuring 12 cm in diameter and the urinary E_1C concentration was $900 \text{ ng mg}^{-1} \text{ Cr}$. The female was given $500 \mu\text{g GnRH}$ at 1200 h on the day of oestrus. Retrospective analysis of urine samples collected from Trial no. 2 indicated that two LH surges (690 and $384 \text{ pg mg}^{-1} \text{ Cr}$) occurred following GnRH injection (Fig. 5c). The first surge was measured in the urine 2 h post-GnRH injection with the second surge detected at 8 h and 50 min. Urinary E_1C concentrations remained elevated throughout the trial (day 0–day 2; 934 , 665 and $478 \text{ ng mg}^{-1} \text{ Cr}$ respectively) until day 3 post-GnRH injection, when the mean concentration measured $59.09 \text{ ng mg}^{-1} \text{ Cr}$. No measurable rise in PdG concentrations occurred during the trial ($43.1 \pm 15.82 \text{ ng mg}^{-1} \text{ Cr}$). Urinary LH concentrations were relatively flat on days 1 and 2 post-GnRH injection reaching baseline ($30.48 \text{ pg mg}^{-1} \text{ Cr}$) on day 3. Ovulation did not occur. Instead, an anovulatory haemorrhagic follicle was diagnosed by rectal ultrasonography on day 3 post-GnRH injection, based on echogenicity of follicular fluid and swirling of the fluid upon ballottement.

Discussion

The Indian rhinoceros appears to exhibit several species-specific reproductive characteristics among the Rhinocerotidae. First, changes in oestrogen concentrations are

readily measured in the urine and/or faeces and reflect cyclical patterns, whereas in the Sumatran, African black and African white rhinoceros oestrogen metabolite analyses have not proven reliable as a means of monitoring ovarian function (Ramsay *et al.* 1987, Hodges & Hindle 1988, Roth *et al.* 2001). Furthermore, studies characterizing the reproductive cycle through the measurement of excreted steroid hormone concentrations and animal behaviour revealed wide intra- and inter-animal variations in the oestrous cycle length of this species (Kassam & Lasley 1981, Kasman *et al.* 1986, Schwarzenberger *et al.* 2000). In this study, ultrasonography was employed for the first time in this species to investigate the dynamics of follicular growth, precise timing of ovulation and the subsequent formation and demise of luteal tissue in relation to endocrine and behavioural patterns. Our findings unveil several additional characteristics that appear specific to this species and also provide information that may help explain both earlier findings by researchers and the difficulties encountered by zoo personnel trying to breed the Indian rhinoceros.

Both female Indian rhinoceroses monitored in the study exhibited strikingly similar oestrous cycle dynamics. The growth of very large follicles appeared characteristic for this species and was not an individual or aberrant cycle specific phenomenon. The diameter of the preovulatory follicle (10–12 cm) in the Indian rhinoceros far exceeds that for other species within the Perissodactyla order. Preovulatory follicles in the domestic horse reach 3.5 to 5.5 cm in diameter (Ginther 1995, Carnedale 1998). Both African species of rhinoceros grow preovulatory follicles similar in diameter to those of the horse (Radcliffe *et al.* 1997, 2001), whereas the Sumatran rhinoceros grows smaller preovulatory follicles measuring just 1.9 to 2.5 cm in diameter (Roth *et al.* 2001). In fact, no other species of mammal is known to grow such large preovulatory follicles. Even a species much larger than the Indian rhinoceros, the elephant, produces follicles that measure only 2.1 cm in diameter (Hermes *et al.* 2000, Hildebrandt *et al.* 2000).

Data in this study indicate that a single wave of follicular development is associated with each Indian rhinoceros oestrous cycle, a finding similar to that reported for African and Sumatran species (Radcliffe *et al.* 1997, 2001, Roth *et al.* 2001). Whereas the Sumatran rhinoceros and domestic horse can develop multiple antral follicles (Ginther 1995, Carnedale 1998, Roth *et al.* 2001), both Indian rhinoceroses in this study developed a single antral follicle on one of the ovaries similar to that which has been reported for the African species (Radcliffe *et al.* 1997, 2001). In many species including the domestic horse, Sumatran, African black and African white rhinoceros, maximum follicle size has proven useful in predicting impending oestrus and ovulation (Ginther 1995, Carnedale 1998, Radcliffe *et al.* 1997, 2001, Roth *et al.* 2001). In contrast, the preovulatory follicle of the Indian rhinoceros achieved its maximum diameter several days

up to 1 week prior to behavioural signs of oestrus. Furthermore, no observable change in the shape of the preovulatory follicle was detected prior to ovulation, as has been reported to occur in the domestic horse, African black and African white rhinoceros (Ginther 1995, Carnedale 1998, Radcliffe *et al.* 1997, 2001).

Ovulation occurred spontaneously in both the female Indian rhinoceroses as it does in the horse, African black and African white rhinoceros (Ginther 1995, Carnedale 1998, Radcliffe *et al.* 1997, 2001), differentiating the Indian species from its Asian relative, the Sumatran rhinoceros, that appears to be an induced ovulator. In both female Indian rhinoceroses, ovulation was consistently followed by formation of a corpus haemorrhagicum. In contrast, the African white rhinoceros appears capable of forming both corpora lutea and corpora haemorrhagica (Radcliffe *et al.* 1997), while corpora haemorrhagica were never observed following ovulation in the Sumatran rhinoceros (Roth *et al.* 2001). We also documented four oestrous cycles in the younger female Indian rhinoceros associated with anovulation and the production of haemorrhagic follicles. Three of the anovulatory cycles occurred from January to May of 2002, with the fourth anovulatory cycle occurring in late December 2002. Anovulatory haemorrhagic follicles have been reported to occur in the domestic horse, African black and African white rhinoceros (Ginther 1995, McKinnon 1998, Radcliffe *et al.* 1997, 2001). In the domestic horse, these follicles are associated with the transition into winter anoestrus and the formation of accessory corpora lutea of pregnancy (Ginther 1995, McKinnon 1998). Researchers have provided evidence that insufficient gonadotrophin stimulation to induce ovulation and insufficient oestrogen production from the follicle itself may be responsible for their occurrence (Irvine *et al.* 2000, Watson *et al.* 2002). Radcliffe *et al.* (1997, 2001) documented the occurrence of anovulatory haemorrhagic follicles in captive African black and African white rhinoceros, hypothesizing seasonal and/or management activities as possible causes for the decline in fertility. Recently, researchers provided endocrine evidence of seasonality in the wild African black rhinoceros (Garnier *et al.* 2002). Through longitudinal faecal hormone analysis, these researchers established a 3 month period of decreased fertility that correlated to changes in the environmental factors of photoperiod, rainfall and temperature (Garnier *et al.* 2002). The Sumatran rhinoceros has also been reported to develop luteinized follicles that were anovulatory, but their occurrence was associated with absence of the mating activity necessary to induce ovulation and was not related to season (Roth *et al.* 2001). Although the reason why the one Indian rhinoceros developed anovulatory follicles is unknown, it is possible they occurred due to reduced photoperiod during the winter months. The fluorescent lights in the stalls housing the female were changed to a fixed 14 h exposure regimen following the December 2002 anovulatory oestrous cycle, and the female successfully ovulated in

the oestrous cycles that followed during the winter of 2003 in Study no. 2. However, despite the fixed light regimen, an anovulatory cycle was again observed in this female in April 2004 (M A Stoops, personal observation). Despite some anecdotal evidence of a photoperiod effect on ovulation, it is more likely that the female is simply in transition from a pre-pubertal state to puberty. The second female Indian rhinoceros ovulated in all oestrous cycles monitored and she was a year older. However, there was a 64 day period during winter (November 1998 to February 1999) in Study no. 1 when ultrasounds were not conducted.

Ultrasound data in this study support previous reports of variable cycle length in the Indian rhinoceros (Kasman *et al.* 1986, Roth & Brown 1999) with the interval between ovulations ranging from 36 to 61 days. Regardless, when mean cycle length was calculated (43 days) it was very similar to that previously calculated based on intervals between oestrogen peaks (Kassam & Lasley 1981, Kasman *et al.* 1986, Schwarzenberger *et al.* 2000). In this study, the longest cycle (61 days) that occurred in the younger female was associated with the formation of a follicular cyst that lengthened an ovulatory oestrous cycle.

The follicular phase of the Indian rhinoceros appears to be the longest among species in the Perissodactyla order. A follicular phase of ~14 days was documented in the two females based on the length of time between the rise of urinary E_1C concentrations above baseline and the day ovulation was detected by rectal ultrasonography. The length of the follicular phase in our study did not differ from those reported by researchers following the rise and fall of oestrogen conjugate concentrations in the urine (Kassam & Lasley 1981, Kasman *et al.* 1986), as concentrations were at baseline ~1 day following ovulation. However, in most mammalian species, growth of the preovulatory follicle can be closely tied to oestrogen production, with the follicular phase an approximate measure of these two highly correlated events. The preovulatory follicle of the Indian rhinoceros could not be reliably predicted until it reached 6 cm in diameter and it was not until the follicle grew to 8 or 9 cm in diameter that urinary E_1C concentrations rose above baseline. Therefore, the length of the follicular phase of the Indian rhinoceros would be considerably longer if based solely on growth of the follicle destined for ovulation. The length of the luteal phase (17 days) reported herein, was similar to the 17–21 days reported in another study of the Indian rhinoceros using urinary hormone analysis (Kasman *et al.* 1986).

The difference in peak urinary E_1C concentrations between the two females could be due to variation in the frequency of urine collections between the two study animals. The younger female was more intensively monitored to determine events around the periovulatory period. Daily urine collections were conducted around oestrus, when peak E_1C concentrations were measured. However, the younger female also had higher baseline urinary E_1C concentrations suggesting an individual specific differ-

ence. In fact, prior studies of the Indian rhinoceros reported similar inter-animal differences in hormone concentrations (Kasman *et al.* 1986, Schwarzenberger *et al.* 2000). Therefore, a real physiological variation in hormone production between the individual females could account for the observed statistical difference in the data.

Oestrous behaviours in the Indian rhinoceros were similar to those observed in the domestic horse and African rhinoceros species (Hafez 1993, Radcliffe *et al.* 1997, 2001). When using oestrous behaviour alone or oestrous behaviour in combination with urinary hormone analysis it was almost impossible to distinguish between ovulatory and anovulatory cycles. At no point during the study was the female put with the male for the purpose of breeding, so we cannot attest to whether she would have exhibited 'standing' oestrous behaviour. However, the oestrous behaviours of anovulatory cycles were of similar duration and intensity to those observed during oestrus of ovulatory cycles. Therefore, rectal ultrasonography was critical in determining if ovulation had occurred 48 h following oestrus. Retrospective analysis revealed that urinary E_1C concentrations on the day of behavioural oestrus in anovulatory oestrous cycles were statistically lower than E_1C concentrations on the day of behavioural oestrus for ovulatory cycles. However, there would be no way to determine whether an oestrous cycle would be anovulatory based on an E_1C concentration collected on the day of behavioural oestrus among a population that exhibits such hormonal variation between individuals and cycles. In prior studies of the Indian rhinoceros, researchers interpreted a decline in oestrogen concentration a short time after oestrus as verification of ovulation (Kassam & Lasley 1981, Kasman *et al.* 1986, Schwarzenberger *et al.* 2000). While E_1C concentrations accurately reflected final follicular growth, a decline in E_1C occurred even with anovulatory cycles. Further confounding the ability to hormonally distinguish between ovulatory and anovulatory oestrous cycles was the formation of haemorrhagic follicles capable of luteinization and progesterone production. All anovulatory cycles were associated with some, albeit erratic, urinary PdG excretion that could be mistaken for a luteal phase. Similarly, in horses, researchers determined that the majority of anovulatory follicles became luteal structures based on elevated progesterone concentrations (McCue 1998, McCue & Squires 2002). Ultrasonographic documentation of anovulatory oestrous cycles may have important implications for captive breeding efforts with Indian rhinoceros, because it establishes the occurrence of infertile cycles that otherwise appear fertile through the traditional methods of oestrous behaviour and endocrine monitoring.

Detailed examination of ovulatory oestrous cycles in the younger female revealed two patterns that differed in length, the number of days that oestrous behaviours were exhibited, and the number of days the preovulatory follicle was sustained at maximum diameter and peak urinary E_1C concentrations. Oestrous cycles associated

with two separate days of behavioural oestrus had statistically longer cycle lengths attributable to a delay in the emergence of the preovulatory follicle and more days spent at maximum diameter prior to ovulation. However, despite these differences, several important consistencies existed during the periovulatory period in the two cycle patterns that may prove useful in breeding decisions and/or developing assisted reproductive techniques in the Indian rhinoceros. With both patterns, the female ovulated within 48 h following a behavioural oestrus. Additionally, a slight, transient rise in urinary PdG concentrations occurred the day following that oestrus and may be useful in predicting ovulation. Even though Kasman *et al.* (1986) were unable to verify ovulation through the methods used in their study, they reported a similar rise in urinary PdG concentrations up to 5 days prior to the decline in urinary E_1C . Progesterone has been implicated to play a role in ovulation in many other mammalian species (Kapustin *et al.* 1996, Zalanyi 2001, Cassar *et al.* 2002, Jo *et al.* 2002, Stouffer 2002).

Changes in rectal temperature and vaginal discharge were also investigated as a means of predicting ovulation in the Indian rhinoceros. In humans, an initial drop in basal body temperature is followed by a rise at the time of ovulation (Davis 1946). In the younger Indian rhinoceros, a $\sim 1^\circ C$ rise in rectal temperature was measured on the day of oestrus for both an ovulatory and anovulatory oestrous cycle. The rise in temperature was attributed to the marked increase in activity level during behavioural oestrus and was not due to the act of ovulation. The occurrence of vaginal discharge in the young female appeared to correlate with progesterone production and was not necessarily verification of ovulation. In ovulatory cycles, the female produced a vaginal discharge shortly after behavioural oestrus, whereas the discharge was delayed in anovulatory cycles, possibly due to slow and/or incomplete luteal tissue formation and function.

In an effort to overcome the challenge of predicting ovulatory cycles, GnRH injections were tested as a means of ensuring ovulation would occur following every oestrus. Early in the validation of the LH assay we discovered that unfrozen urine samples were essential, as quantifiable decreases in LH concentration were measured with each freeze-thaw cycle that the sample experienced. Following this discovery, all urine samples collected from the female were aliquoted into two vials, with one kept at refrigerator temperature to use in the LH EIA. During most of the oestrous cycles, the female would urinate up to 8 times during a 24 h period, but around oestrus, when an LH surge would be expected, the female urinated with intense frequency (up to 30 times during a 24 h period). Additionally, it appeared that the LH surges of the Indian rhinoceros were cleared through the kidneys and into the urine rather rapidly. Therefore, long term monitoring of urinary LH during all oestrous cycles was not deemed to be logistically feasible because all urine excreted would have had to be collected and analysed. However, the establishment

and use of the LH assay was essential to document the female's response to GnRH challenges.

Results from the two GnRH trials indicate that administration of 500 µg GnRH on the day of peak oestrus does stimulate LH release from the pituitary of the female Indian rhinoceros that can be measured in the urine approximately 2 h following injection. However, GnRH did not induce ovulation. Although the oestrous cycle associated with GnRH Trial no. 1 was ovulatory, ovulation did not appear to be due to GnRH administration. The oestrous cycle associated with Trial no. 1 had two separate occasions of behavioural oestrus and administration of GnRH during the first oestrus did not advance ovulation and prevent the occurrence of a second behavioural oestrus. Because an LH surge followed the GnRH injection, it is unlikely that failure to induce ovulation was due to differences in the recognition, binding and response of the Indian rhinoceros GnRH receptors to the form of GnRH that was administered. More likely, the female failed to ovulate due to insufficient maturation of the preovulatory follicle at the time of the first behavioural oestrus. Despite the GnRH-induced LH surge during the first oestrus, pituitary stores were not depleted as an endogenous LH surge occurred several days later, followed by ovulation. Because a 500 µg GnRH dose tested in Trial no. 1 resulted in an LH surge, the same dosage was tested during a second trial to confirm consistency of the elicited response. Results from Trial no. 2 confirmed that an LH surge occurs in response to this treatment and can be detected in the urine ~2 h post-injection. In fact, LH concentrations appeared slightly higher in this second trial. Even so, the cycle was anovulatory, suggesting that a single GnRH injection will not induce ovulation of a follicle destined to be anovulatory. This finding is consistent with those documented in the horse. Not all mares responded with ovulation of a dominant follicle following administration of GnRH during the anovulatory season (Ginther & Bergfelt 1990) and the majority of attempts to induce ovulation were associated with the formation of a luteinized anovulatory follicle (McCue 1998, McCue & Squires 2002).

The reproductive physiology of the female Indian rhinoceros appears to be uniquely species-specific in many ways and by no means simple to comprehend. Results from the present study help elucidate some of this species' reproductive characteristics largely due to the integration of ultrasonography with the more traditional methods used to study reproductive function. Through the use of ultrasonography, we were able to document the unique follicular dynamics associated with the oestrous cycle of the female Indian rhinoceros. In addition, we established patterns of follicular growth, timing of ovulation in relation to oestrus, hormonal measures that may predict ovulation in this species, and documentation of infertile cycles that would otherwise not have been apparent through traditional measures. These results provide a more complete and detailed understanding of the reproductive

physiology of the Indian rhinoceros that may aid in the development of assisted reproductive technologies and/or provide better methods for accurately timing natural breeding attempts.

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