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TECHNICAL REPORT

Combined Serial Ultrasonography and Fecal Progestin Analysis for Reproductive Evaluation of the Female White Rhinoceros (*Ceratotherium simum simum*): Preliminary Results

Robin W. Radcliffe,[1]* Nancy M. Czekala,[2] and Steven A. Osofsky[1]

[1]*Fossil Rim Wildlife Center, Department of Animal Health Services, Glen Rose, Texas*
[2]*Center for Reproduction of Endangered Species, San Diego, California*

Ultrasonographic examinations of one multiparous 33-year-old female southern white rhinoceros (*Ceratotherium simum simum*) resulted in documentation of the animal's estrous cycle, elucidation of the timing of ovulation in relation to estrus, and ultrasonographic evidence of endometritis and associated early embryonic death. The preovulatory follicle was observed to change in shape from spherical to pear-shape (n = 3) and to reach a mean follicular diameter of ~30 mm (n = 4) in the 48 hr preceding estrus. An ovulation site in the location of the preovulatory follicle was observed to occur within 24 hr postbreeding on one occasion. Both pregnancies monitored in this female in 1995 were lost by day 28 postovulation, with collapse of the embryonic vesicle documented via ultrasound. Ultrasonographic evidence of endometritis was observed in this female and was characterized by small quantities of anechoic intrauterine fluid collections (5–20 mm diameter) in late diestrus (n = 4, mean day observed was 20.5 days postovulation, with a range of 18–24 days). Fecal samples collected at the time of ultrasound were evaluated via radioimmunoassay for progesterone metabolites. A substantial rise in fecal progestins was not identified until 7–9 days postovulation. This study illustrates the value of combining the complementary techniques of ultrasonographic "mapping" of events with fecal hormone assay to enhance the accuracy of reproductive monitoring. Zoo Biol 16:445–456, 1997.

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*Correspondence to: Robin W. Radcliffe, D.V.M., Animal Health Services, Fossil Rim Wildlife Center, P.O. Box 2189, Rt. 1, Box 210, Glen Rose, TX 76043.

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INTRODUCTION

This study represents the first long-term evaluation of reproductive dynamics in a white rhinoceros through ultrasonographic imaging and documents that combining serial ultrasonography with fecal progestin analysis measurably improves the accuracy of reproductive monitoring.

The estrous cycle of the southern white rhinoceros has previously been reported as ranging from 32–70 days [Hindle et al., 1992; Schwarzenberger et al., 1994] as determined by urinary and fecal hormone assays, respectively. Previous research efforts investigating the reproductive biology of the rhinoceros have primarily involved serum, urine, fecal, and saliva hormone assays [Kassam and Lasley, 1981; Kock et al., 1991; Hindle et al., 1992; Schwarzenberger et al., 1993, 1994, 1996; Czekala and Callison, 1996]. Other investigators have utilized ultrasonographic evaluation of immobilized and chute-conditioned rhinos, but have focused on its feasibility and the documentation of reproductive anatomy [Schaffer and Beehler, 1990; Adams et al., 1991]. The perceived intractability of the rhinoceros has limited research efforts in the past, and consequently there has been no consensus on basic reproductive parameters in these species. The utilization of a free-stall chute [Radcliffe et al., 1995, 1996] has facilitated reproductive research efforts in both the white and black rhino species by allowing serial transrectal ultrasound examinations without subject sedation.

MATERIALS AND METHODS

Animals and Ultrasound Technique

One multiparous wild-caught southern white rhinoceros ("Macite," studbook no. 147, estimated birthdate 1963) was maintained in a large outdoor enclosure with access to a barn at the Fossil Rim Wildlife Center and was evaluated by transrectal ultrasonography and fecal hormone assay approximately three times per week during 1995. The observational period reported here encompassed nearly a complete year, with the first ultrasound exam on January 11 and the last exam on December 6, 1995. The ultrasound procedure was performed by chute-conditioning the subject, using food as a positive reinforcement for entering and remaining in a free-stall chute [Radcliffe et al., 1995] (Fig. 1). The nonsedated rhinoceros was free to enter or leave the chute at any time, but was usually amenable to remaining in the chute for the ultrasound examination while eating. The operator performed the exam from behind a safety wall and could immediately leave via the exit area if the rhinoceros decided to back out of the chute. The chute design eliminated anxiety previously noted when subjects were completely confined in a more traditional stall.

Transrectal ultrasonography was performed with a portable scanner (Aloka 500V, Aloka, Wallingford, CT) equipped with a 5-MHz convex-array transducer (Aloka Model UST-935N-5, 47° scan angle). The transducer was originally designed for transcutaneous use and was modified for the purposes of the present study. Equipment modifications to facilitate ovarian examinations in the white rhinoceros included a custom 10-foot transducer cable and an extensor. The extensor, formed of PVC

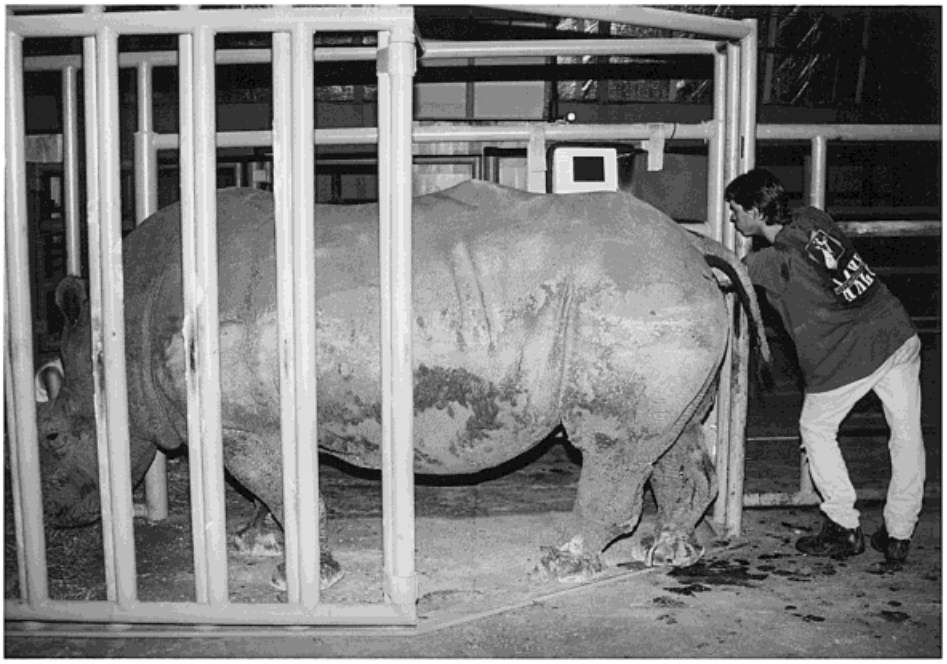

Fig. 1. Photograph of the free-stall chute design illustrating ultrasonographic reproductive examination of the nonsedated rhinoceros.

pipe and reshaped via thermal manipulation to house the transducer, was needed to image consistently the subject's left ovary, which was beyond the operator's unassisted reach. The extensor measured 1.22 m in length and was angled as illustrated in Figure 2 to facilitate rectal manipulations needed for visualization of the left ovary. The right ovary was routinely imaged without the use of an extensor. During the first 30 examinations when the ultrasonographic technique was being developed and various extensor designs were being tested, only 53% of examinations resulted in observation of both ovaries. However, for the duration of the study, using the final extensor design, identification of both ovaries was successful on 86 of 94 attempted ovarian exams (92%). All ultrasound examinations were recorded on Hi-8 professional videocassette (Sony Recording Media of America, Montvale, NJ) for later evaluation.

Macite was primarily kept with two other white rhino females and one male in a large (~10 acre) pasture. Another male was located in close proximity in an adjacent corral. Male and female rhinos were held separately in a barn for variable periods of time when needed because of inclement weather. Estrus in the white rhino was defined as receptivity of the female to approaches by the male. The rhino females were observed daily while outdoors for behavioral signs of estrus and breeding activity. The other female rhinos held with Macite have been evaluated via the methodologies described. Those results are not included here: one subject was a 2-year-old prepubertal female, whereas the other female exhibited no signs of estrus and no ultrasonographic evidence of cyclicity.

The rhinoceros belongs to the order *Perissodactyla*, or "odd-toed" ungulates, which includes the horse and tapir. The equine species was used as a model for interpretation and evaluation of ultrasonographic information in this study [Ginther,

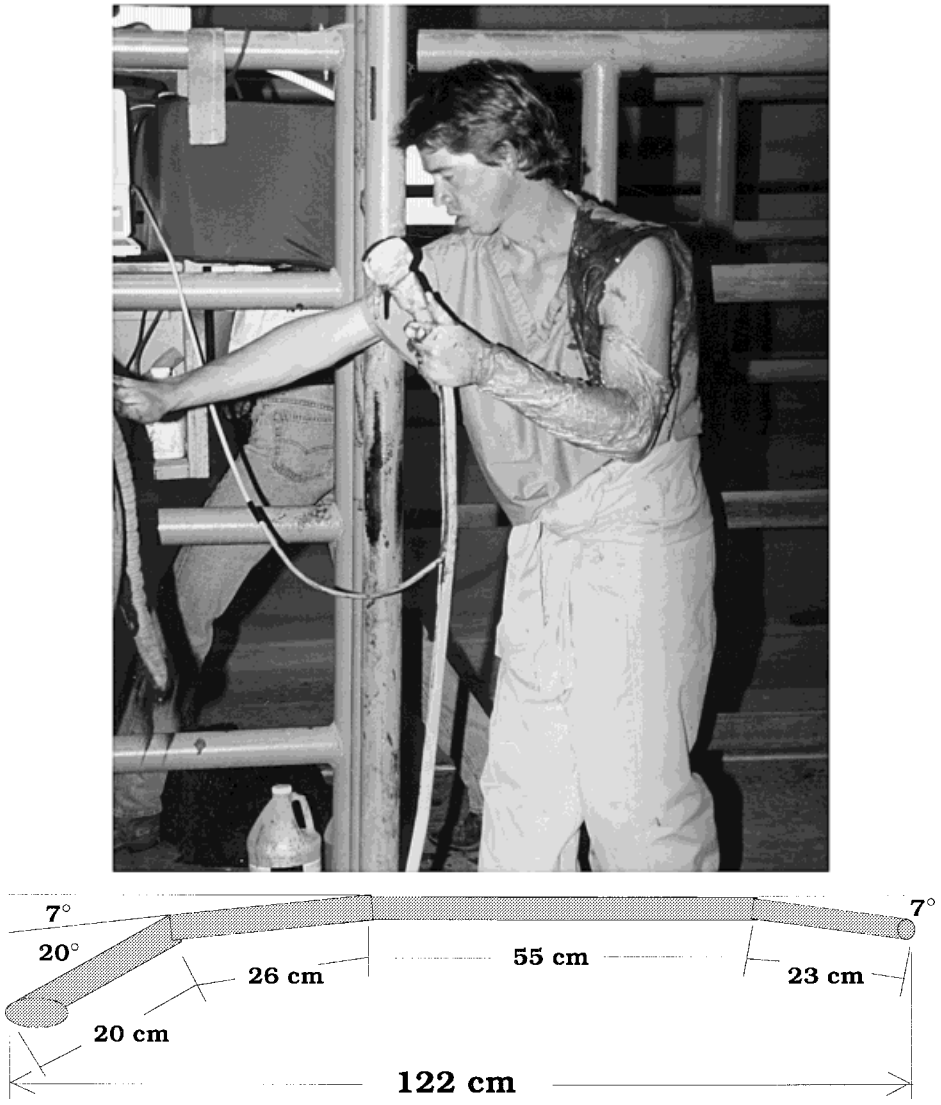

Fig. 2. Photograph and accompanying drawing detailing the design of the PVC extensor used to facilitate ultrasonographic evaluation of the left ovary.

1992, 1995; McKinnon and Carnevale, 1993], and this comparative approach has facilitated understanding of rhinoceros reproductive biology despite some obvious anatomical differences.

Radioimmunoassays

Fecal samples were collected directly from the rectum prior to each ultrasonographic examination. Approximately 50 g of feces were placed in a plastic bag and frozen at –20°C until analysis. Each fecal sample was lyophilized to dryness and sifted to remove vegetation debris. A 0.2-g aliquot of dried, cleaned feces was placed into a 16 × 150-mm glass test tube, mixed with 2 ml distilled water, and extracted

with 5 ml anhydrous diethyl ether (Mallinckrodt, Paris, KY). The ether extract was dried and resolubilized in 1 ml ethanol. The progesterone assay utilized 0.01 ml of extractant. Extraction efficiency of added tritiated progesterone was 55%. Progesterone metabolites were assayed using radioimmunoassay techniques employing a monoclonal antibody produced against 4-pregnen-11-ol-3,20-dione hemisuccinate:BSA (1:32,000; provided by Dr. Jan Roser, University of California, Davis). Tritiated progesterone (10,000 cpm/0.1 ml, Dupont NEN, Boston, MA) was used to compete against standard progesterone (7–1,000 pg, Sigma, St. Louis, MO). Phosphate-buffered saline, pH 7.0 with 1% gelatin, was used as diluent.

The assay was incubated overnight at 4°C and terminated by the addition of 0.25 ml charcoal dextran solution with incubation for 30 min, followed by centrifugation. The supernatant was counted for 2 min in a scintillation counter using Ultima Gold (Packard, Meriden, CT) scintillation fluid. Cross-reactivities for this antisera have been previously described [Wasser et al., 1994]: 96% 5α-Pregnane-3β-ol-20-one; 36% 5α-Pregnane-3α-ol-20-one; 7.4% 5β-Pregnane-3α-ol-20-one; and 4.8% 5β-Pregnane-3α, 17α-diol, 20α-one. All other steroids tested were <0.2%. Interassay coefficient of variation for control fecal samples was 22%. Serial dilution of rhino fecal samples as compared to the progesterone standard curve was parallel ($r = 0.997$). Typically, the antibody dilution bound 34% of added tracer, nonspecific binding was 3%, and assay sensitivity was 15 pg/tube. All progestin concentrations are reported as ng/g dry weight of the fecal samples.

RESULTS

The reproductive anatomy of this female correlated well with the gross and ultrasonographic rhinoceros anatomy previously reported [Schaffer and Beehler, 1990; Adams et al., 1991; Godfrey et al., 1991; Schaffer et al., 1994]. The cervix was highly convoluted and measured ~70 mm in diameter and 150 mm in length. The uterine body diameter was 40–45 mm. Uterine horn diameters measured 30 mm at the corpus-cornual junction and 20 mm in their cranial location adjacent to the ovaries. The ovaries were oval and measured ~60 mm × 40 mm depending on structures present. The uterine echotexture did not change significantly in this female rhino over the course of this study.

Two nonconceptive cycles were observed and two longer conceptive periods with identified early embryonic loss were documented via ultrasonographic examination. Nonconceptive cycle lengths of 31 and 35 days were documented, with fecal progestins remaining elevated on each occasion for 19 and 22 days, respectively. As in the cow and horse [Pierson and Ginther, 1988; Ginther, 1992, 1995], two types of luteal gland ultrasonographic morphologies were identified. One type, the corpus hemorrhagicum, forms when the collapsed follicle fills with blood and becomes organized. The resulting central clot remains ultrasonographically detectable throughout luteal life. The other type is identified by its uniform ultrasonographic appearance with no central clot and presumably lacks the hemorrhagic event that results in clot formation. These two types of luteal glands were formed in an approximately even ratio, with three of five (60%) luteal structures forming a central clot ($n = 5$ diestrous periods). In the two cases of early embryonic loss observed to date, two longer conceptive periods of 73 and 78 days were identified (Fig. 3).

Typical estrous behaviors lasted less than one 24-hr period and included urine

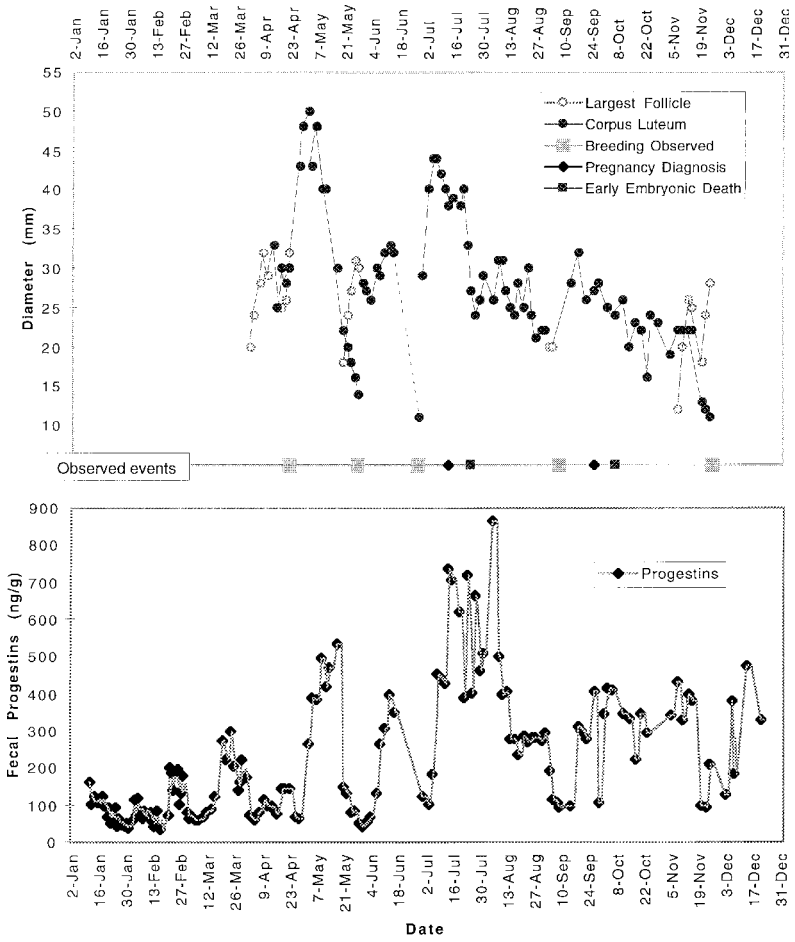

Fig. 3. Fecal RIA progestin profile (ng/g dry weight) encompassing the 11-month study period (bottom) and its correlation with behavioral and (ultrasonographically monitored) reproductive events (top). Dashed line indicates no ultrasound exams.

squirting similar to that observed in the mare, curling or lifting of the tail to the side, and allowance of close approaches by the male with eventual "standing" behavior as the male placed his head on the female's rump. The color of the vaginal mucosa was noted to change from pale pink in diestrus to red and hyperemic ~3 days before through the 2 days following estrus. The follicular phase was short, lasting ~9 days, with the preovulatory follicle reaching a mean diameter of ~30 mm (range, 28–32 mm measured at longest diameter; n = 4) in the 48 hr prior to estrus. A noticeable change in follicular shape was detected in three of four preovulatory periods, with the follicle changing from spherical to pear-shape in the 48 hr preceding estrus (Fig. 4). The event of ovulation (day = 0) was identified by confirmation of collapse or evacuation of a mature preovulatory follicle (Fig. 4). Although the actual event of follicular evacuation was not observed, an ovulation site was identified within 24 hr postbreeding on one occasion. The formation of a dominant preovulatory follicle was accompanied by a quiescent contralateral ovary (n = 4, with one ovulatory pe-

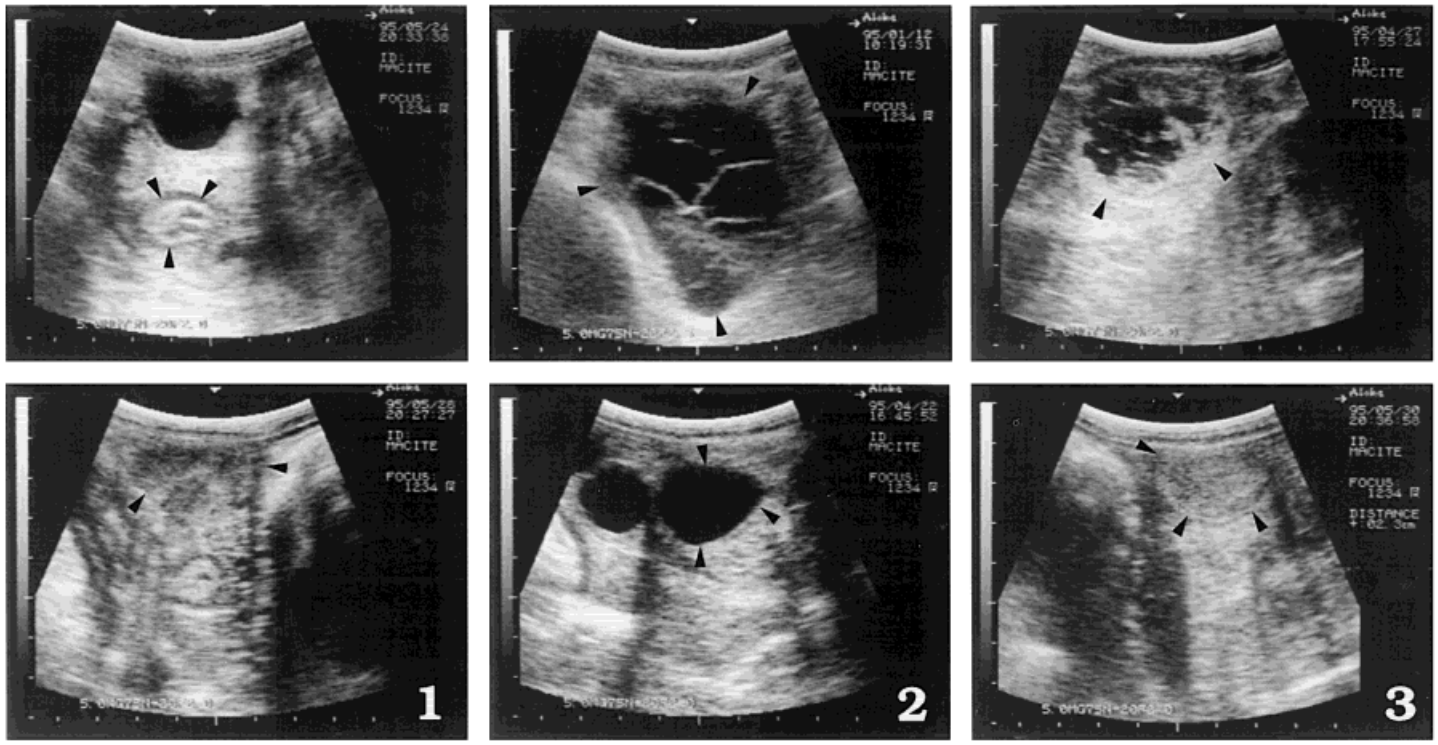

Fig. 4. **1:** A maturing preovulatory follicle 3 days prebreeding, with a small regressing luteal structure from the previous ovulation (arrows, top), and the ovulation site, 1 day postbreeding (arrows, bottom). Axes are marked in 1-cm intervals. **2:** A large follicular structure possibly analogous to the anovulatory hemorrhagic follicles documented in domestic equids (arrows, top). For comparison, note the pear-shape preovulatory follicle (arrows, bottom). Axes are marked in 1-cm intervals. **3:** The two types of luteal structures identified include the "central clot" form or corpus hemorrhagicum (top), and the homogeneous corpus luteum (bottom). Axes are marked in 1-cm intervals.

riod not studied). The first four ovarian events (April through early September) were documented on the left ovary, with September through early December yielding one event involving the right ovary.

During the first several months of ultrasonographic evaluation (January–March), there was some cyclic ovarian activity, but there were no observed ovulatory events until a short cycle in early April. This period of presumed anestrus was later confirmed by low levels of fecal progestins via RIA analysis (Fig. 3). In addition, at the time of initial exam of this female (January 11, 1995), a large ovarian structure was observed on the left ovary. This structure measured 60 mm in diameter and appeared to be a follicle with luminal fibrous bands that quivered upon ballotment as if in a gelatinous state (Fig. 4). This structure appeared to be analogous to an equine hemorrhagic follicle and was considered to be an anovulatory structure as is often observed in horses during the transitional phase, that period in the mare characterized by erratic ovarian activity preceding reentry into the ovulatory season. The time of formation of this structure was not known, but once identified, it was seen to disappear gradually over 10–15 days.

The diagnosis of pregnancy was made by day 15 postovulation by ultrasonographic visualization of the embryonic vesicle. The embryo proper was identified as early as day 23, with visualization of the fetal heartbeat as early as day 26 (Fig. 5).

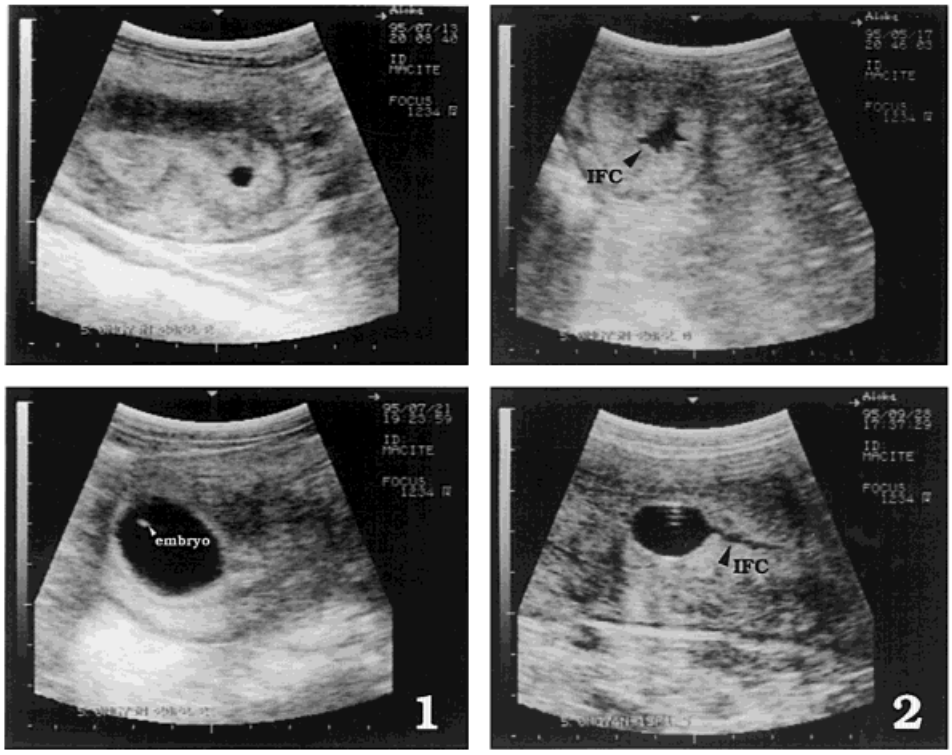

Fig. 5. **1:** A 15-day embryonic vesicle (top) and the 23-day embryonic vesicle with identifiable embryo proper (labeled, bottom). Axes are marked in 1-cm intervals. **2:** An intrauterine fluid collection (IFC) observed on day 24 of estrous cycle (top) and evidence of impending EED with visible fluid (IFC) surrounding a 19-day embryonic vesicle (bottom). Axes are marked in 1-cm intervals.

Embryo orientation within the vesicle appeared to be inconsistent, and fixation of one vesicle was suspected on initial examinations (days 15 and 17) in the right corpus-cornual junction. However, embryo mobility was observed on subsequent exams. In both pregnancies, early embryonic death (EED) was confirmed by the collapse or disappearance of the vesicle and embryo proper by day 28. Endometritis had been suspected in this female, based on the observation of small quantities of anechoic intrauterine fluid collections (IFC) (5–20 mm diameter) in late diestrus. Intrauterine fluid collections were observed in the four luteal phases that were comprehensively monitored, with the IFC identified at a mean of 20.5 days postovulation (range, 18–24 days). The IFC was observed surrounding the embryonic vesicle in one of the pregnancies (Fig. 5).

Figure 3 illustrates the changes in fecal progestin concentrations as they relate to events noted on ultrasound and through behavioral observations. Fecal samples were monitored throughout 1995. Following ovulation without conception, the progestin concentrations increased substantially 7–9 days after the documented ovulation and remained elevated for 19 and 22 days before returning to baseline concentrations. Ovulation occurred at the nadir of the fall of fecal progestin concentrations. Following conception, the progestins followed a similar pattern as in the nonconceptive cycle; however, concentrations remained elevated past the 19–22-day luteal rise (Fig. 3). The corpus luteum structure persisted 42 and 48 days following the documented collapse of the embryonic vesicle, and the progestins remained elevated for ~44 days (n = 2) following the collapse. The regressing luteal structures remained identifiable during the upcoming follicular phase (Fig. 4), whereas fecal progestins were observed to decline.

DISCUSSION

The perceived intractability of the rhinoceros had limited the use of ultrasonography prior to this study. The serial use of ultrasound without the need for sedation has provided the authors with a unique opportunity to evaluate reproductive parameters of the rhino over time. This study represents the first documentation of the precise timing of ovulation in a rhinoceros as it relates to observed behavior and is the first requisite step toward the application of advanced reproductive techniques such as artificial insemination that may be needed in the future. Managed breeding decisions finally can be based on objective reproductive assessments of individual animals instead of conjecture. More research involving additional females is needed. However, significant technical conclusions still can be drawn from this study, especially since the ultrasonographic and fecal hormone evaluations encompassed a yearlong period and a number of duplicated reproductive events.

Several important findings were documented during the preovulatory period. As has been reported for the horse [Ginther, 1992], both the increasing follicular diameter and the change in shape of the preovulatory follicle from spherical to pear-shape may be valuable predictors of impending ovulation in the rhinoceros. The observed inequality in the side of ovulation, with four of five ovulatory events identified on the left ovary, was more similar to the horse than the cow [Ginther, 1992].

The interovulatory interval of ~33 days for a nonconceptive cycle is in close agreement with the behavioral estrous cycle length of 38 days reported at the San Diego Wild Animal Park (Rieches, personal communication), where the most suc-

cessful captive propagation of this species has occurred. Hindle et al. [1992] reported a cycle length of 32 days in the southern white rhinoceros based on evaluation of urinary estrogens and progestins. Estrous cycle lengths of ~10 weeks, however, identified via fecal enzyme immunoassays (EIA) alone [Schwarzenberger et al., 1994] have been reported and contrast with results reported here. Interestingly, embryonic death can be associated with prolonged maintenance of the corpus luteum as visualized ultrasonographically in both cattle [Kastelic et al., 1988] and horses [Bergfelt et al., 1992].

The events of pregnancy, followed by early embryonic death, and the associated observation of intrauterine fluid collections in late diestrus could not have been documented without application of serial ultrasonographic techniques in this rhinoceros. The spherical vesicle shape and embryo proper measurements identified in this female are similar to those described for a 27-day black rhino pregnancy [Adams et al., 1991]. The IFCs, observed as anechoic collections in the uterine lumen and surrounding one of the vesicles, are suspected to be an inflammatory exudate in response to uterine irritation. In mares, a strong correlation has been shown between the identification of IFCs in late diestrus and impending EED [Ginther, 1992], and both IFCs and EED were more extensive in old than young mares [Carnevale and Ginther, 1992]. The timing of embryonic loss (prior to day 28) in this rhinoceros (n = 2) also supports a diagnosis of endometritis: a healthy uterine environment is considered essential to equine embryo viability beyond day 28 due to the increasing metabolic demands of the conceptus [Ball, 1993]. In both pregnancies, irregular changes in embryo orientation and mobility occurred over time, different from what is seen with a healthy equine conceptus [Ginther, 1992, 1995]. Perhaps this was an indicator of imminent embryonic loss. A thorough documentation of embryo orientation and development is needed in a normal rhino pregnancy to evaluate if the observations reported here are indeed an indication of impending EED in this species.

Fecal hormone assay via RIA was essential for confirmation of morphological changes and to understanding underlying physiology. The delay in fecal progestin rise following documentation of ovulation is an important finding. The 7–9-day time period from ovulation to the first substantial rise in fecal progestins is in close agreement with the 5–10-day period observed in the black rhinoceros [Schwarzenberger et al., 1993]. The mean gastrointestinal transit time in the white rhinoceros is reported to be 65 hr [Foose, 1982], so the lag cannot adequately be explained by this alone. Further work identifying the first progesterone rise in *serum* will be needed in the rhinoceros to interpret any lag phase that may be inherent with fecal hormone analysis as has been done for equids [Schwarzenberger et al., 1992]. The ability to ultrasonographically monitor events and relate observed ovarian changes to underlying reproductive endocrinology will help validate both methodologies in this species.

CONCLUSIONS

1. Although findings in this white rhino female corroborate those of Hindle et al. [1992] as well as behavioral reports, we cannot generalize about the estrous cycle length of the white rhino species with data from one 33-year-old reproductively abnormal animal.

2. Combining the complementary techniques of ultrasonographic “mapping” of

events with fecal hormone assay can enhance the accuracy of reproductive monitoring. Concurrent fecal hormone assays confirmed ultrasonographically identified reproductive cycle dynamics, and this served to validate both methodologies. Without ultrasound, however, delineating the precise timing of ovulation, differentiating between early pregnancy and a nonpregnant luteal phase, and identifying early embryonic loss would prove difficult.

3. The preovulatory period was characterized by a change in follicular shape from spherical to pear-shape and by growth of this dominant follicle to a diameter of ~30 mm in the 48 hr preceding estrus.

4. In relation to observed breeding behavior, ovulation was identified to occur within 24 hr postbreeding on one occasion. Ovulation was detected ultrasonographically 7–9 days before a substantial rise in fecal progestins.

5. A number of similarities to the horse were documented in this white rhino female.

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