Gestational Monitoring of Eastern Black Rhinoceros (*Diceros bicornis michaeli*) Through Ultrasonography, Serum and Urine Hormone Quantification, Fetal Assessments and Girth Measurements.

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Introduction

With only approximately 500 Eastern black rhinoceros (*Diceros bicornis michaeli*) surviving in the wild, the captive population needs to sustain its current numbers (International Rhinoceros Foundation website, 2002). At the recommendation of Rhinoceros Taxonomic Advisory Group and in cooperation with Addo Elephant Sanctuary, South Africa, The Kansas City Zoo imported two wild-caught females in 1996 and 1997 to provide valuable genetics to the captive population.

The monitoring of estrus cycles in Eastern black rhinoceros has been done by several researchers (Loskutoff et al., 1983; Ramsay et al., 1987; Hindle et al., 1992; Czekala and Callison, 1996; Schwarzenberger et al., 1996; Berkeley et al., 1997; Roth, 1999). In 1999, we initiated a research project to monitor the estrus cycle via serum and urine hormone derivatives while correlating these values with behavioral observations. Our knowledge of behavioral parameters associated with reproduction enabled us to successfully introduce animals for breeding. Upon confirmation of conception, we made the decision to expand our collection period to encompass pre-conception, conception and the entire gestation period for one of the females.

To yield a complete profile, and broaden our knowledge of this species' gestational progression, we expanded our conditioning program to include more husbandry behaviors. Ultrasonography has been utilized to evaluate reproductive status in a variety of species (Adams et al., 1991; Radcliffe et al., 1999). However, this procedure
has not been routinely used in eastern black rhinoceros. We utilized operant conditioning practices and a confinement chute to enable us to conduct weekly transrectal and transabdominal ultrasonography procedures. Staff also conducted daily fetal assessments as a means to evaluate fetal viability. Finally, for comparison, the dam's anterior and posterior girth measurements were taken on a monthly basis.

The data from urine and serum hormone analysis, coupled with ultrasonography, girth measurements and fetal assessments has broadened our gestational monitoring capability for this endangered species.

Materials and Methods

Urine samples

Collection:

To avoid contamination that may affect analysis, animal was conditioned to either eliminate on front barrier for midstream collection, or desensitized to use of collection apparatus for this process. These midstream urine samples were generally captured in the morning by staff. Midstream samples were preferred, but if staff was unable to capture sample, we did aspirate some samples from the floor. Urine samples were collected twice weekly, labeled, and frozen immediately until analyzed. The Smithsonian Laboratory

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(National Zoological Park, Conservation and Research Center, 1500 Remount Road, Front Royal, VA, 22630-5972) performed all urine sample analysis.

Creatinine analysis:

Urine samples were analyzed for creatinine (Crt) content to help compensate for variation in fluid intake and output (Hindle et al., 1992). All hormone concentrations are indexed as mg Crt.

Hormone analysis:

All samples were evaluated for estrone (EC) and pregnanediol-3-glucuronide (PdG). Estrogen levels are cited as ng EC/mg Crt. Progesterone levels are cited as ng PdG/mg Crt. All samples were correlated to breeding date and its appropriate stage of gestation.

Serum samples

Collection:

Serum samples were obtained via venipuncture by veterinary staff, in alternating forelegs, utilizing a 19g X 1" butterfly catheter. Samples were placed in serum separator tubes, centrifuged, serum extracted and frozen (at -80° C) prior to analysis. Initially serum samples were collected weekly, but frequency of collection increased during last stage of gestation. The Smithsonian Laboratory performed all serum sample analysis.

Hormone analysis:

All samples were evaluated for estradiol (E2) and progesterone (P4). Estrogen levels were cited as pg/ml values and progesterone levels were cited in ng/ml.

Fetal assessments:

On a daily basis staff conducted fetal assessments as a means of evaluating fetal viability (Illustration One). As part of our morning routine, staff would position animal parallel with the barrier to perform this evaluation. The palm of hand was placed on abdomen along the flank and, using a stopwatch, timed the interval between the calf's kicks. Staff charted the interval between the start of a session and the first kick and between first and second kick. Staff also made note of nature and force of kick (abrupt, distinct, blunt, etc.) as a means to gauge fetal activity and viability.

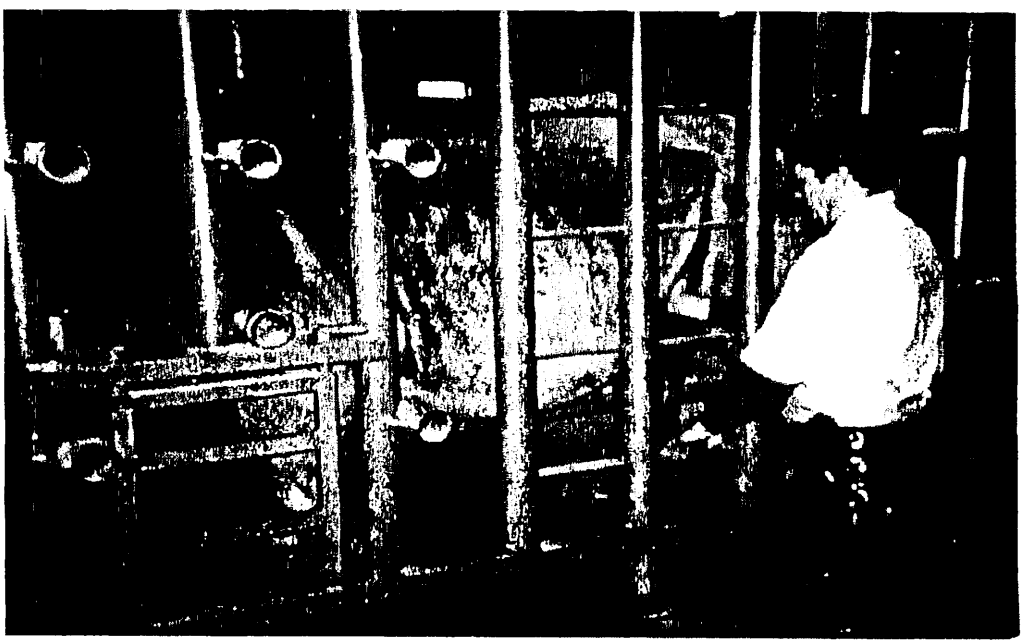


Illustration One: Staff conducting daily fetal assessments.

Girth measurements:
To quantify another physical change in the dam throughout gestation, staff measured her girth on a monthly basis (Illustration Two). A tape measure was utilized to acquire the anterior (mid-back) and posterior (anterior to flank) measurements. The animal was conditioned to placement of tape, tightening of device, measurement, and subsequent movement of tape to the site for the second measurement.

Illustration Two: Staff performing monthly girth measurements

Ultrasonography:
Operant conditioning techniques and the use of positive reinforcement have broadened the types of husbandry practices that can be performed (Priest, 1990; Kinzley, 1993; Desmond and Laule, 1994; Thorne and Whalen, 1996). By utilizing these techniques, staff was able to condition the animal to enter the chute and permit both transrectal and transabdominal ultrasonography procedures to be performed (Shaffstall, 2000). Staff was responsible for all training, via positive reinforcement, for the restraint chute and ultrasonography procedure. We also desensitized the animal to the movement and presence of additional equipment, machinery and personnel. Rhinoceros staff was responsible for initial positioning and confinement of animal, while maintaining position to permit imaging via transrectal and transabdominal ultrasonography. The animal tolerated these procedures to the extent we were able to permit local broadcast stations to witness this procedure and convey this knowledge to the public. It should be noted that The Kansas City Zoo is the first institution to provide visual access to this procedure through the use of Internet technology. Public knowledge of the process was from afore-mentioned media broadcast, but specific day and time of procedures were posted on web page so interested individuals could view this unique procedure via web-cam.

Transrectal:

Knowledge of the reproductive tract and its associated structures were reviewed and provided the necessary information prior to initiation of the ultrasonography procedures (Godfrey, 1991). Transrectal ultrasonography enabled veterinary staff to detect and measure ovarian structures and evaluate/document reproductive status (Adams et al., 1991). We utilized an Ausonic impact ultrasound unit (Ausonics Impact VFI, Universal Medical Systems, Bedford Hills, New York, 10507) with a 3.5 mHz linear probe for the transrectal procedure.

Transabdominal:

Due to the dense body mass and size of these large herbivores, no one has successfully performed a transabdominal procedure. However, we were successful at imaging a fetus, throughout gestation, with a 2.5 mHz variable sector probe (Illustration tTree). The region in which this procedure could be done is relatively small when compared to the size of the animal. We were consistently able to image fetus within a five inch diameter region along the lower flank of the female by utilizing stationary positioning (Illustration Four).

Illustration Three: Transabdominal ultrasonography procedure.

Illustration Four:Close up of region where transabdominal ultrasonography was performed.

Results

Serum and urine hormone quantification:

Figure one illustrates the urine estrone conjugate (EC) detected in each sample. As mentioned, all values are indexed with creatinine (Crt) to account for fluctuations in fluid intake and output. All samples are correlated with the appropriate stage of gestation with zero denoting date of breeding. The correlation of serum estrogen, estradiol (E2), with stage of gestation is documented in Figure two. Previous research has indicated that a decline in estrogen derivative is associated with the collapse of the follicle at time of ovulation (Kasman et al., 1986). This trend is difficult to determine since there was a limited collection time prior to conception. However, we did see cyclic pattern of estrogen (E2) concentration dropping for three cycles prior to conception (-52, -36 and –16 days). At this time estrogen sample analysis is incomplete and concludes at day 152 (E2) or day 237 (EC), respectively.

Figure one. Correlation of urine estrogen (EC) with stage of gestation

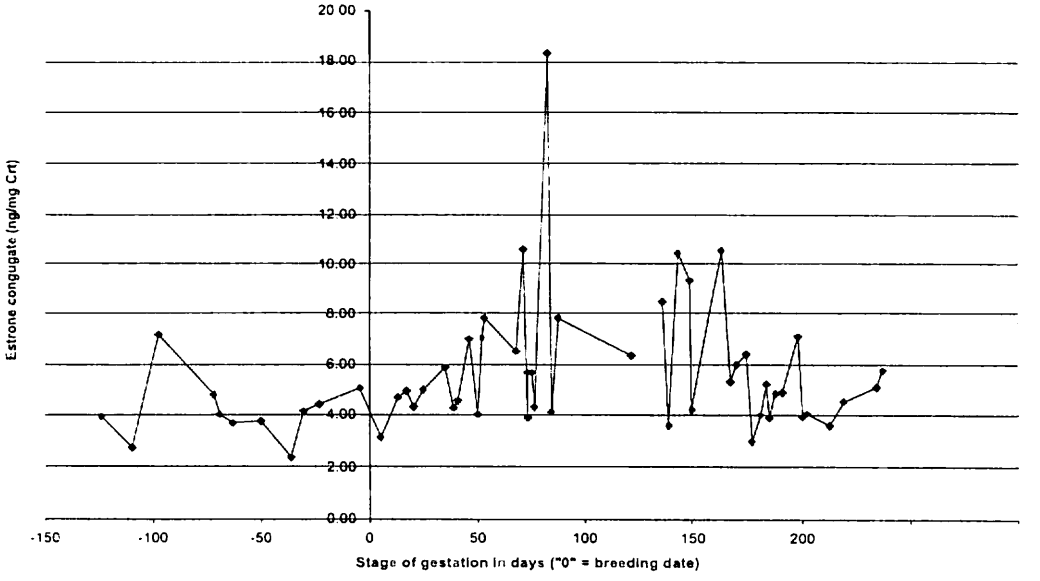


Figure two. Correlation of serum estrogen (E2) with stage of gestation

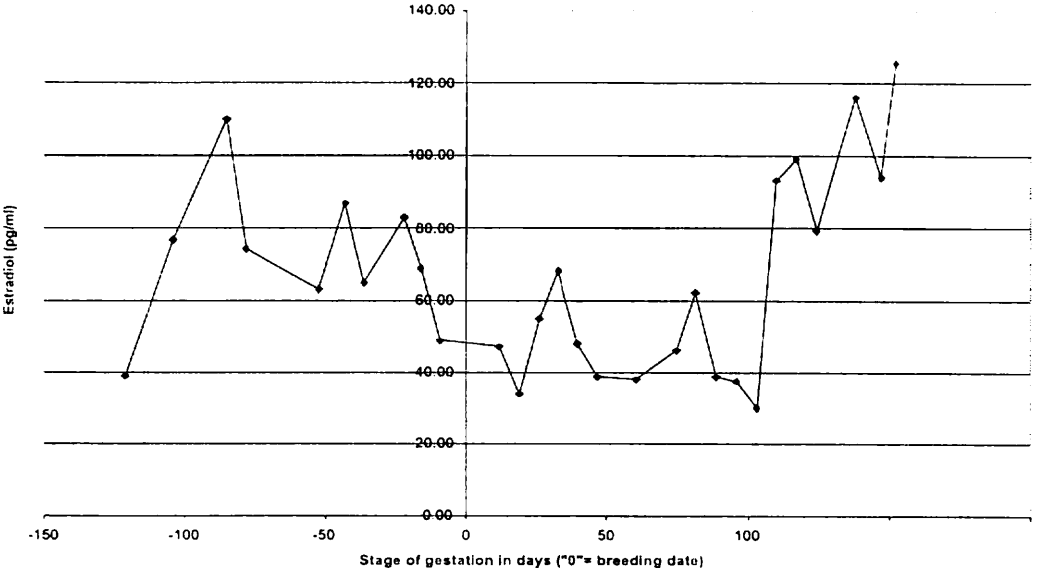


Figure three correlates the progesterone derivative excreted in urine, pregnanediol-3-glucuronide (PdG), with the appropriate stage of gestation. Previous research has indicated that PdG became measurable after 120 days post-breeding (Ramsay et al., 1987). Our results closely followed the trend found in this earlier research. Prior to 125 days post breeding, the average concentration was 12.70 ng/mg Crt. However, after that time, values reflected an average 18.6 fold increase over previous concentrations. The average value during this interval was 235.60 ng/mg Crt. In this particular female, we did not see any PdG concentrations below 64.81 ng/mg Crt after 125 days post-breeding. Prior to 125 days there was no PdG value above 15.63 ng/mg Crt. We did observe a brief decline in PdG concentration 48 days prior to parturition but concentrations returned to their former levels. A secondary decline in PdG concentration was observed one day prior to parturition.

Figure three. Correlation of urine progesterone (PdG) with stage of gesta

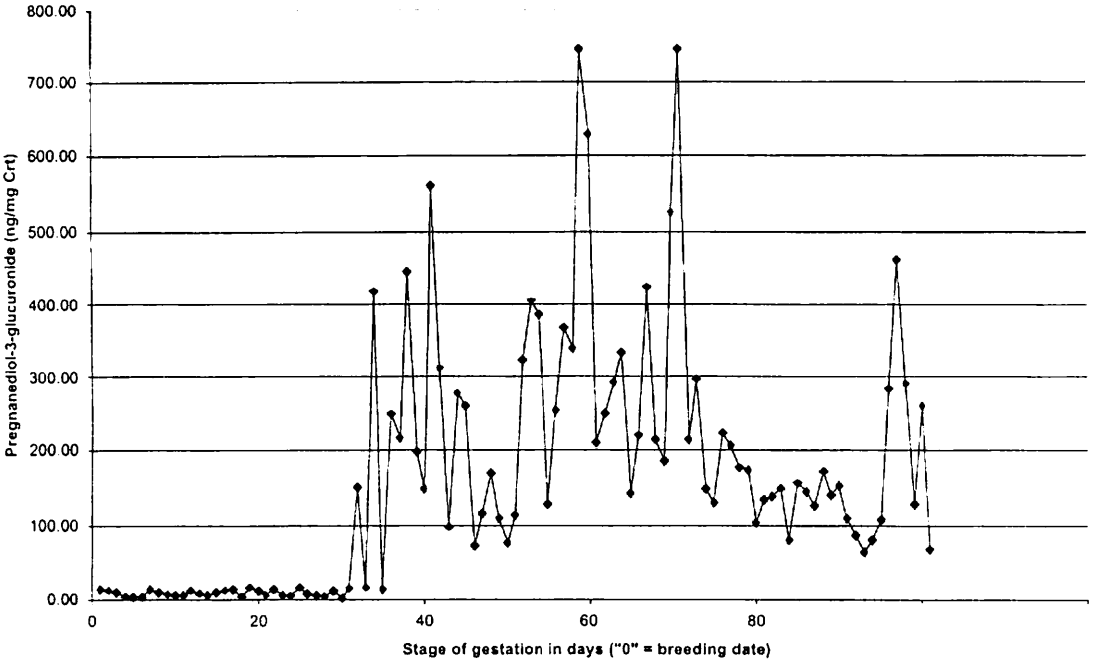
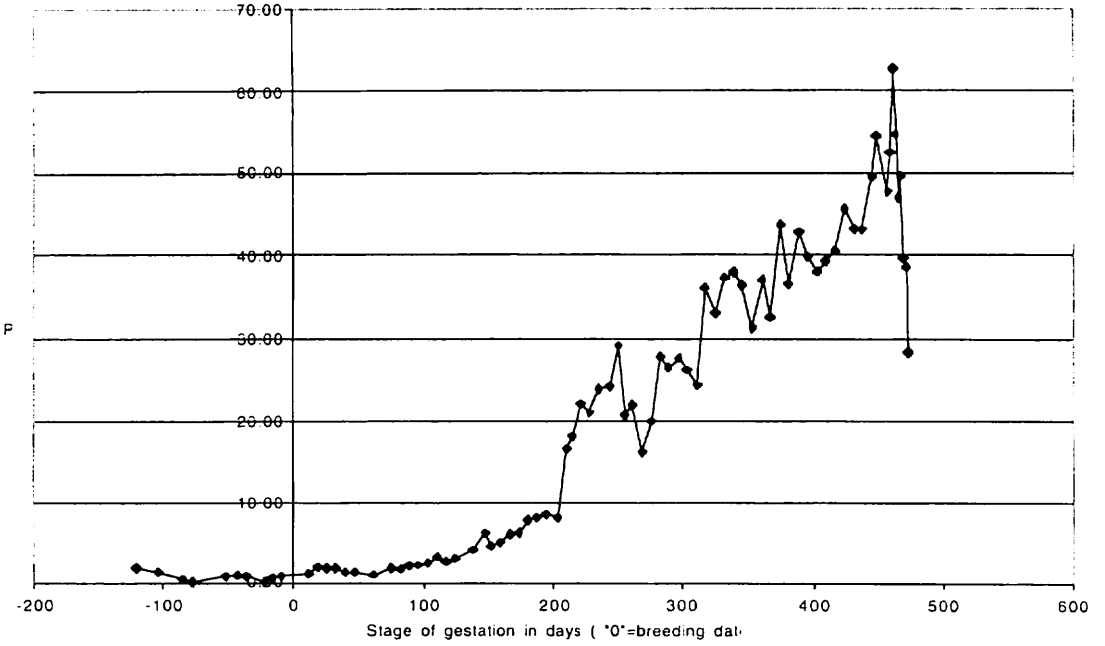


Figure four shows the concentration of serum progesterone (P4) throughout collection period. An elevation of serum progesterone levels after conception has been documented in prior research (Kock et al., 1991). As reflected in the graph, progesterone concentration showed significant increase between days 125 and 160, but increased dramatically at approximately 210 days post-breeding. Prior to this time, P4 levels averaged 3.05 ng/ml which was higher than fluctuations observed during normal, cyclic activity. After 210 days the concentration increased to an average level of 35.26 ng/ml. But, P4 levels peaked at 12 days prior to parturition then initiated a steady decline in concentration after that collection.

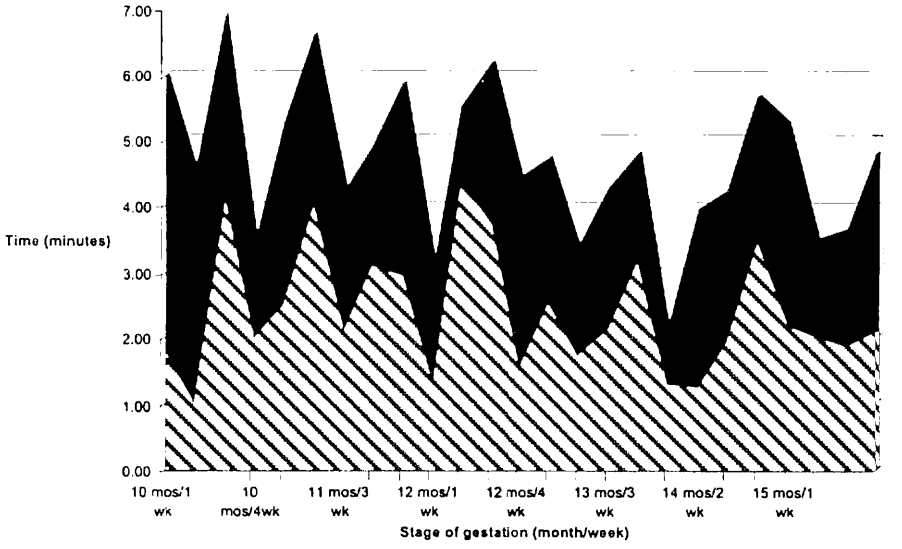
Figure four: Correlation of serum progesterone (P4) with stage of ge



Fetal assessments:

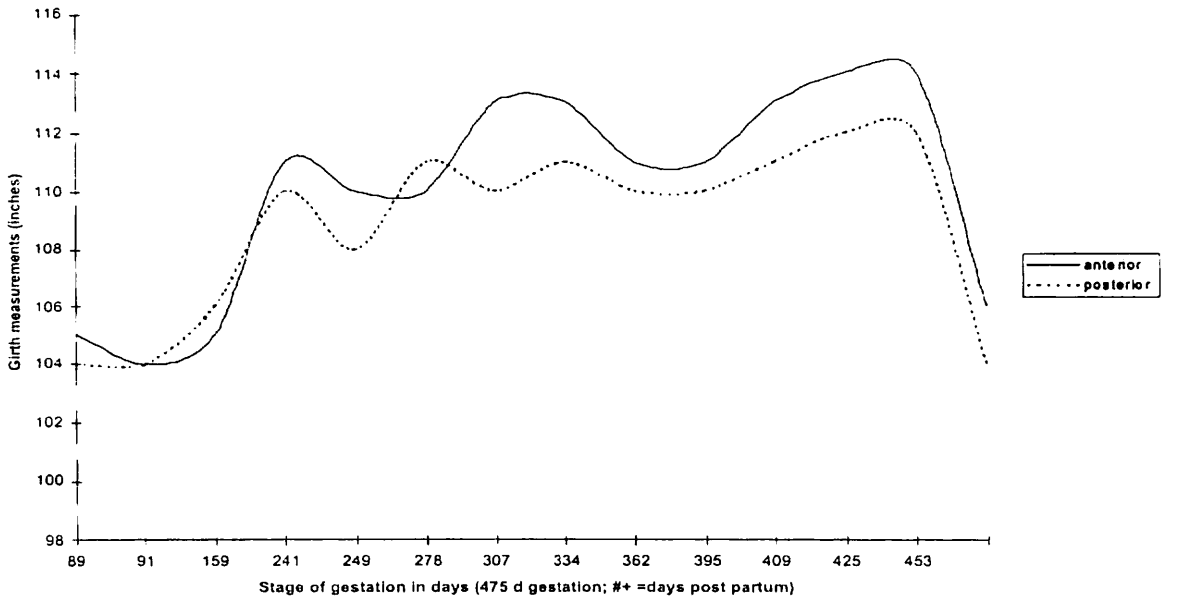
Fetal assessments were initated in the third quarter of gestation period. We observed great variability with these daily evaluations, as reflected in Figure five. For easier comparasion, the daily times were arranged into weekly averages. We do not feel the time intervals themselves will be able to be utilized in a comparative manner in future pregnancies, however, the daily determination did provide insight into fetal viability. Staff noted variation in the force and "feel" of the kicks. Initially, calf's movements were sharp, distinct and in relatively rapid succession. In later stages the kicks felt more like a blunt push or a rolling sensation across the palm of your hand. It should also be noted that all assessments were done on the animal's left side, since staff was unable to feel any movement on the opposite side of body.

Figure five: Graph of fetal assessment overview (on weekly basis)



Girth measurements:
Figure six depicts the measurable physical changes in girth observed in this female. There was some fluctuation observed, but generally a steady increase occurred over time. The anterior girth measurement increased a total of 10 inches (25.4cm) throughout gestation. Whereas posterior girth remained relatively stable until the last stage of gestation, then increased. Posterior girth increased approximately eight inches (20.32cm) in diameter.

Figure six: .Graph of girth changes associated with stage of gestatio



Ultrasonography:
Ultrasonography was performed with a portable unit equipped with variable 2.5 mHz sector probe (Ausonics Impact VFI, Universal Medical Systems, Bedford Hills, New York, 10507) for transabdominal ultrasound, and a 3.5 mHz rectilinear probe transrectal ultrasound. Transrectal imaging was consistent with previous investigations into this procedure (Adams et al, 1991; Radcliffe, 1999; Roth, 1999)

For the transabdominal procedures, warmed ultrasound gel was applied during multiple sessions without the use of the probe. Once comfortable with each session, more time was spent conditioning for the next procedure. Ultrasound imaging was performed on a weekly basis at an average depth of 18†– 22cm (~7-8.5 in.). A small "window" provided consistent imaging. This "window" was caudal and ventral to the extent of the rib cage, just above the level of the stifle. Images were only obtained on the left side, although attempts were made to image the right side of the maternal abdomen. Images could not be obtained on ventral midline, or higher on the abdominal wall. On occasion images could not be obtained due to preclusion by intestinal contents and peristalsis. Transabdominal ultrasound appeared to stimulate fetal movement.

Most of the appendicular anatomy (ears, nares, feet, tail) and several maternal reproductive structures (placenta, umbilical cord, uterine wall, amnion) were visualized, measured and monitored throughout pregnancy. Viability of the fetus was assessed with each session through a combination of ultrasound and

tactile sensation of the abdomen. During the last trimester of pregnancy, placental mineralization was observed and documented. Mineralization increased as gestation progressed.

Discussion

The girth measurements are routinely used in the management of our eastern black rhinoceros collection. In this instance, it provided insight into one of the physical changes observed during gestation.

The fetal assessments provided staff with an awareness of fetal viability on a routine basis. This information, communicated to our veterinary department, contributed to our means of monitoring fetal development. We do not feel the time parameters will be generally applicable, however, the actions proved to increase staff awareness, and evaluation, of fetal viability.

The comparative analysis between urine and serum hormone levels indicate that either means can be used to evaluate reproductive status. This will benefit institutions that are unable to perform venipuncture, but could collect or aspirate a urine sample. In this female, the progesterone derivative pregnanediol-3-glucuronide (PdG) excreted a levels above 15.63 ng/mg Crt, after 125 days post-breeding interval was consistent with pregnancy. Additionally, serum progesterone, P4, detected at levels above 8.56 ng/ml after 210 days post-breeding were consistent with pregnancy. The same determinations can be made utilizing serial saliva samples (Czekala and Callison, 1996) or serial fecal samples (Berkeley et al., 1997; Schwarzenberger et al., 1996) to determine progesterone levels.

Operant conditioning techniques have greatly expanded the husbandry behaviors that can routinely be performed by staff. Most institutions have expanded their management programs to accommodate the use of ultrasound technology. This technology has provided a means of evaluating reproductive status based on measurement of reproductive structures. Pregnancy has been diagnosed, via rectal ultrasound, as early as 16 – 27 days post-breeding (Radcliffe et al., 1999; Roth, 1999). Additionally, there are preliminary results that suggest the measurement of fetal eye and foot diameter can be used to determine fetal age (Radcliffe et al., 1999). This could be invaluable to institutions that are unable to perform these procedures on a routine, comparative basis or may not have definitive breeding dates. In the same manner, the presence of placental mineralization may be used to indicate approach of parturition. In this female, placental mineralization was detected at approximately two months prior to parturition.

In conclusion, we wish to stress that none of the afore-mentioned practices could have been completed without a dedicated staff. The additional time required for the training, measurements and assessments contributed to our monitoring capability for both dam and fetus throughout the entire gestation period. The knowledge gained has been shared with other institutions to improve their management of this endangered species.

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