Greater One-Horned Rhinoceros (*Rhinoceros unicornis*) Artificial Insemination Program . . . from a keeper's perspective

Wendy Shaffstall, Senior Ungulate Keeper

Cincinnati Zoo & Botanical Garden

The development of an artificial insemination program for the greater one-horned rhinoceros (*Rhinoceros unicornis*) is a unique collaboration of scientists and dedicated rhino professionals. Since its formation in 1981, the Center for Conservation and Research of Endangered Wildlife (CREW) has been instrumental in applying scientific principles and technology to saving endangered species. But, the greater one-horned rhinoceros artificial insemination program also required the skills, knowledge and dedication of the keepers on the project team to ensure its success.

Historically, the use of ultrasonography has provided a means of directly imaging and measuring ovarian structures while characterizing reproductive events (Adams, 1991). It has been found that the reproductive management of captive African rhino species can be enhanced through the application of transrectal ultrasonography (Radcliffe, 1999). So, in 1999, reproductive physiologists sought to use this technology to correlate follicular development with urinary hormone metabolite monitoring of the estrogen derivative (estrone conjugate) and progesterone derivative (pregnanediol-3-glucuronide) in the greater one-horned rhinoceros (Roth, 2000; Stoops, 2003)).

As the program evolved and our knowledge of this species increased, we found several unique features of their reproduction. The estrus cycle is approximately 45 days in length but can range from 35 – 60 days (Roth, 1999; Stoops, 2004, 2005). During the cycle, the greater one-horned rhinoceros develops several small follicles on both ovaries that continue to grow until one reaches approximately 6 cm in size. At that point in time, the follicle becomes "dominant" and continues to increase in size while the other follicles regress (Stoops, 2004). When the follicle reaches the 8 – 9 cm, the urinary hormone concentration of estrogen will begin to rise above baseline value, but these values can vary among individuals (Roth, 2000; Stoops, 2004). The estrogen serves to act upon the uterine environment to make it suitable for implantation should fertilization occur. The final follicle size will reach 10 – 12 cm, which is the largest of any mammal studied and can be maintained up to a week prior to signs of behavioral estrus (Stoops, 2004). It is when the estrogen concentration begins to increase that the female begins the "follicular phase" of her cycle. During this fourteen day phase, olfactory and phermone stimulation is critical so keepers provide exposure to male feces and monitor her reaction (Stoops, 2004). But, since we do not have a male at our facility, we must utilize feces frozen and provided to our institution for this purpose. The astute observations of the keepers are critical to determining when the female begins showing signs of behavioral estrus. Regardless of the ovarian changes and concentration of rising estrogen, one female may show strong behavioral estrus signs and another may be more subdued. Generally, behavioral estrus signs may be manifested by loss of appetite, increasing pacing activity, "whistling" vocalizations, "squirting" urination patterns and vulvular "winking". These signs should occur at day 12 of the "follicular phase", but variances in this time frame are common. The scientists must rely on the keepers' ability to pick up on these subtle nuances in behavior, movement or appearance that only comes with perceptive observations to indicate a variation (Radcliffe, 1999).

To ensure efficient communication and continuity, keepers complete daily observation sheets specifically for the greater one-horned rhinoceros. Sheets have sections designated appetite, samples collected, urination pattern, vocalizations, discharge, and behavioral observations. Samples are collected midstream or aspirated off the floor, labeled and refrigerated until analyzed. The concentration of hormone derivatives in these samples is correlated with ultrasound data, follicular measurements and keeper observations to determine the stage of her cycle.

During females' "follicular phase", samples must be collected daily to enable close monitoring of hormonal fluctuations that may indicate impending ovulation. As mentioned previously, the estrogen concentration will begin to rise above baseline during the "follicular phase", then peak for several days, then decline; while the levels of progesterone continue to rise (Stoops, 2004). The high levels of progesterone act on the uterine endometrium to enhance the formation of cells for implantation (Bronson, page 67). The ovulation process itself is induced by a dramatic surge in luteinizing hormone to cause the ovulation of the mature follicle (Bronson, page 69). Ovulation generally occurs within 48 hours following behavioral estrus and ultrasound images reveal the formation of a corpus haemorrhagicum one day later (Stoops, 2004). Luteinization is critical to the estrous cycle conclusion, the formation of the corpus luteum. A functional corpus luteum, will produce rising levels of progesterone foster and provide the proper uterine environment for an embryo.

The keepers' role in the project is all-inclusive. We are responsible for urine sample collection, behavioral observations, documentation, training for ultrasonography, conditioning for phlebotomy and venipuncture procedures, desensitization to vaginal stimulation, training for insemination procedure itself and participating in all insemination procedures. All training is done using operant conditioning principles based on positive reinforcement. We are fortunate that the restraint system we utilize for ultrasound is in a location that females must pass through regularly for shifting purposes. Since the animals were already acclimated to this area, we just had to refine our training and condition them to the confinement of the restraint itself. As with any training pro-

gram, the animals dictated the rate of progression; and, as expected, each progressed at a different rate. During this process we focused on reinforcing calm demeanor and attitude while confined, then proceeded to acclimate them to the movement of the access panel needed to permit the transrectal ultrasound procedure. The next step in our progression was getting them accustomed to a person being in position behind them, then progressing to tactile contact and tail manipulation. Desensitization to the sound and movement of the equipment cart was done while the animal was in the restraint area and was rewarded, as before, for calm attitude and demeanor. The final phase was the conditioning to the rectal insertion of finger, then hand, then arm, and finally arm with hand holding the probe into the rectum. And, due to the large body mass of the greater one-horned rhinoceros, we needed to ensure acceptance of the probe extender that is utilized to lengthen the reach of the examiner. With all of these steps successfully conditioned, routine ultrasounds were done on each of the two females three times per week to monitor estrous cycles. In addition, as a cycle progressed, the frequency of these examinations were increased to enable us to more accurately pinpoint when ovulation could occur. Therefore, when ovulation became imminant, project team members needed to be available to perform ultrasonography procedure at varying times during the day and/or evening. The solid foundation of positive reinforcement for compliance with these procedures enabled the reproductive physiologist to record and document valuable changes in ovarian structures while a project team member handled and positioned the animal during the examination so the ultrasounds could be done in a safe manner.

The monitoring of the females' ovarian changes during the estrous cycle and diligent observations by project team members are essential to the timing of insemination to ensure it is close to ovulation. When the program started three years ago, animals had to be fully immobilized to perform the inseminations, and aside from the apparent risks associated with the immobilization itself, the anesthetics could influence the estrus cycle. We had been trying to desensitize animals to tactile contact of the vulva and vestibule, with limited success until 2006. We recognized the female who had been bred by a male, was more tolerant of this conditioning and the tactile desensitization than the second female with no breeding history. In addition, we found that neither female was responsive to any vulvular contact or manipulation except when she was in the "follicular phase" of her cycle. After discussing these species-specific peculiarities, the project team decided not to do any desensitization until a female entered the 14 day "follicular phase" of her cycle. During this phase the team would use this opportunity and coordinate conditioning sessions between keepers and reproductive physiologist. To avoid contamination, all equipment we used was sterile and the perineal area was cleaned thoroughly before any vulvular desensitization was done. Initially, we used sterilized pipette tubing (with non-spermicidal lubricant that would be used for the procedure) to desensitize females to the tactile stimulation of the reproductive tract. As you can imagine, progress was slow, but the dedication of all team members finally came to fruition. Through numerous trial and error techniques, we finally determined the idiosyncrasies of each female that would result in their cooperation for this particularly invasive procedure that was not evident or needed in any other previously conditioned procedures. Once we tailored our insemination conditioning to suit the animals' needs, progress was rapid.

Originally, both females were conditioned using an optic-equipped gastroscope. The use of this equipment required two additional personnel due to its complexity; one person to control insertion of the scope and second to control the air/sterile water infusion system. The reproductive physiologist was responsible for guiding the scope via optics and performed the insemination itself. Prior to the use of the gastroscope, we did attempt procedure just using the sterile pipette tubing inserted into the tract and progressed to the insemination site. However, in this species of rhino, their reproductive anatomy makes an insemination difficult. In this species, as you enter the vagina the tracks goes up and over the pelvic girdle before you reach the cervix that consists of not only cartilaginous rings, but folds of tissue as well. And once the cervix is penetrated, we want to inseminate in the same horn as the maturing follicle is present. The female who had been bred by a male was ultimately more tolerant of pipette being held and directed to the insemination cite, so she progressed at a more rapid rate than her conspecific. The training for pipette procedure proceeded slowly, as with the conditioning for transrectal ultrasound and gastroscope, but eventually we were able to condition one of the females to insertion of an arm into the reproductive tract with pipette grasped within the palm of the hand and she no longers requires the gastroscope for insemination procedures. The second female has not progressed to this point and her insemination procedures still require the gastroscope.

Communication between individuals is limited to essential information, but a steady dialogue is kept between the project team member managing the rhino and the reproductive physiologist coordinating the insemination procedure. To date, we have successfully performed the transcervical insemination technique in both greater one-horned rhinoceros females. The one female is now able to be inseminated via hand-held pipette, but the second one still requires the gastroscope for the insemination procedure.

Spermatozoa from rhino species have been successfully electroejaculated and cryopreserved using a standard hoofstock semen freezing protocol (Roth, 2001; Stoops, 2006; Stoops, pers. communication). All spermatozoa is evaluated for evidence of morphologic defects, total sperm number and motility prior to storage in subzero temperatures (Blanchard, pages 155, 157). The aforementioned spermatozoal characteristics are re-evaluated after thawing to determine quality and if there was any damage as a result of cryopreservation. At the conclusion of each insemination procedure, we verify the semen deposition site by transrectal ultrasongraphy and subsequent ultrasound(s) are done to verify ovulation.

If fertilization does not occur, there will be an accumulation of fluid in the uterine lumen approximately 15 days after ovu-

lation. Generally, this fluid accumulation coincides with the reduction in the level of progesterone signaling the female has concluded this cycle (Stoops, 2004). But, if fertilization did occur, the embryonic vesicle could be apparent as early as 15 days after ovulation as in other rhino species (Stoops, pers. communication). The fertilization of the egg will result in the corpus luteum being maintained and it will dictate consistent production level of progesterone. In African rhino species, a decline in progesterone levels are commonly seen prior to parturition, and, to a limited extent, in Asian species as well (Shaffstall, 2002; Stoops, pers. comm.). In addition, we hope to assess development of placental mineralization as another factor to be used in narrowing the parturition timeline (Shaffstall, 2002). With confirmation of fertilization, parturition timelines will be finalized and gestational guidelines will be implemented.

This project, however trying at times, has served to foster a sense of comradery between the reproductive physiologist and her project team while all of us are gaining valuable knowledge about this endangered species. We would like to share this knowledge with our colleagues and offer this summation:

Estrus cycle is approximately 45 days (but range from 35 – 60 days)

Multiple follicles can develop, but until one reaches approximately 6 cm in size it does not become "dominant"

Maximum follicle size can reach 10 – 12 cm in size

Common behavioral estrus signs generally are: loss of appetite, increased pacing activity, "whistling" vocalizations, "squirting" urination pattern, vulvular "winking"

Correlation of urinary metabolite levels with ultrasonography monitoring of ovarian structures provides a more complete portrayal of reproductive events

Anovulatory cycles can occur, but may not be evident if there is only reliance on urinary metabolite monitoring to determine reproductive status

During 14 day follicular phase, we provide females exposure to at least male feces for olfactory and pheromone stimulation

Artificial inseminations can be done with spermatozoa that has been collected via electroejaculation and frozen using hoofstock semen freezing protocol

Artificial insemination site is intrauterine deposition into the horn of the ovary with maturing follicle

As with other rhinoceros species, embryonic vesicle could be evident as early as day 15, if fertilization has occurred

Greater one-horned rhinoceros can be conditioned to accept artificial insemination without anesthesia with a patient, but persistent, keeper staff that openly communicates between themselves and reproductive physiologist in charge of the project.

Greater one-horned rhinoceros artificial insemination project team:

Dr. Monica Stoops, Reproductive Physiologist (CREW @ Cincinnati Zoo and Botanical Garden)

Randy Pairan, Ungulate Department Head Keeper

Ungulate keepers: Steve Yelverton, Wendy Shaffstall, Renee Carpenter, Jason Faessler

Sincere thanks to:

Dr. Monica Stoops who encouraged our interest, essential involvement, championed the keeper's integral role in this project; Dr. Terri Roth, for being supportive of this project and the vital need for keeper(s) involvement; Ed Spevak, Conservation Program Manager for Mammals, who patiently accommodates the schedule fluctuations that must occur to enable us to participate in this ground-breaking project.

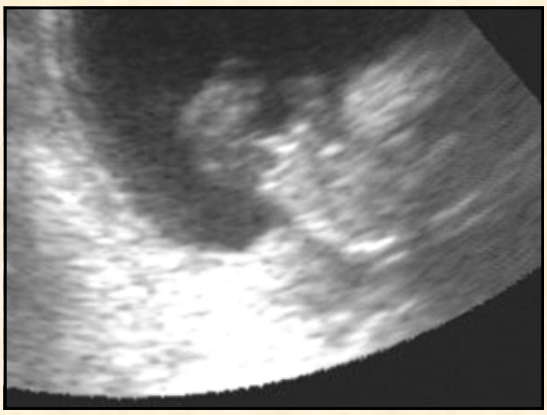
And finally, enough thanks can not be given to my fellow team members who support each other during the trials and tribulations, and put their lives on hold as each cycle progressed toward the insemination time-table.

Article was originally published in Animal Keeper's Forum

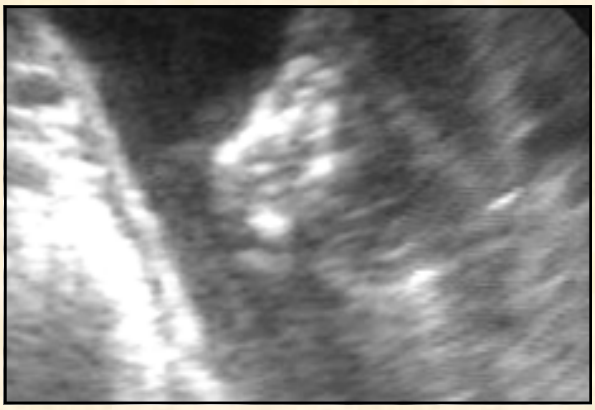
(vol. 34, no. 5, 2007) and re-printed with permission of AAZK, Inc.

Project update:

Cincinnati Zoo and Botanical Garden is pleased to announce the first, successful conception via artificial insemination in this rhinoceros species. The insemination took place in August, 2006 and since fertilization we have monitored implantation, embryo development and the subsequent formation of fetus. We continue to monitor and document fetal growth and development so it will benefit other insititutions who are attempting to unravel the reproductive mysteries of this endangered species.



88 Days Gestation



104 Days Gestation

Please contact Wendy Shafstall at wsrhino@peoplepc.com for Bibliography details

A Guinea Pig and Seven Rhinos

Cydney Peterson

Rhino/giraffe care specialist and Animal behavior management coordinator

Racine Zoo

"So, do they eat meat?" a zoo guest asked me one day as I chatted with eager children asking me questions all at once. The query took me a back two fold. Not only was this a question that I have never had to answer before, but the guest inquiring was an adult. A rapid succession of thoughts poured through my brain as I prepared to answer her. "Surely this was just a question she was asking for her children," I thought. "Could someone really grow up and live a third of their adult life not knowing that rhinos are strict herbivores?" I quickly came to the conclusion that this woman truly did not know what the black rhino before her ate. Later that afternoon I filed through my mental Roladex® of rhino facts and experiences and thought about my role both as a keeper and an educator. I thought about the many people who settle into their jobs/careers (including myself at times, and this was one of those times) and forget that the general public does not know what keepers know. Even though the woman with the meat question earlier in the day clearly knew less than I did about rhinos, I realized that at four and a half years into my zoo career I too still had many unanswered questions about these prehistoric-looking creatures.

Thanks to that one naive rhino question, I was once again thirsting for more information from colleagues with more years of experience. I turned to the Rhino Keeper Association (now IRKA) website for more resources. As a professional member of the Association, I frequented this resource for up and coming news. As the website progressed, so did the goals of the IRKA, including a new program, the Keeper Professional Development Program. The website read "…(the) program will provide rhino keepers the opportunity to expand their knowledge and husbandry experience. This program has been designed to enable rhino keepers to work at other institutions to familiarize themselves with different management styles." This was *IT* ! This program was exactly what I needed to aid my career growth.

In October of 2006, my Director at the Racine Zoo requested that all proposals be submitted for the following year. I immediately wrote down workshops and programs that interested me. I narrowed my choices down to two, the IRKA Program and the SOS Rhino Sumatran Rhino Survey in Borneo. What the heck, I thought, why not write up proposals for both opportunities, I have nothing to loose and everything to gain. To my amazement, the Racine Zoo's President and CEO (Jay Christie) and Director of Conservation, Education and Animal Welfare (Eric Hileman) granted me the support I needed to attend both of my professional development/travel opportunities. As the buzz began wearing off, I focused on contacting the appropriate people. Of the four hosting institutions (Cincinnati Zoo and Botanical Garden, The Wilds, Columbus Zoo and Aquarium, and San Diego Zoo's Wild Animal Park) I applied to spend a week at the Cincinnati Zoo and Botanical Garden to gain additional knowledge of the species that I care for (Eastern black) and the species that I was crossing my fingers to encounter in the wild (Sumatran rhino, Borneo subspecies). Randy Pairan (Head Keeper, Ungulate Department at the Cincinnati Zoo and Botanical Garden and IRKA Board of Directors member) guided me through the application process. Upon my first discussion with Randy, I learned that I was the first keeper to participate in the program; theoretically, I was the guinea pig and Randy's team, the laboratory. Randy and I discussed the best time for my participation and settled on April 8th through the 12th.

As the weeks counted down, my anticipation of gathering new information steadily increased. What I didn't know was that I was not going to be the only rhino keeper learning from the experience. I thought that everyone at Cincinnati would flood