There are currently ten animals at Dvur Kralove including one of mixed sub-specific ancestry. The oldest t and dominant cow — which originally came from Britain (Knowsley) ——has had offspring sired by three different males, the first of which was a southern white. The hybrid from this latter mating was born in 1977, while the pure-bred northern white rhino calves were born in 1980 and 1983. This same female was in oestrus in the summer of 1986 and was sequestered with a northern white bull; she can be expected to reproduce in 1987. Owing to the technical difficulties of shifting animals, the other females are without bulls during oestrus periods, and none have reproduced. The chief constraint at Dvur Kralove is the extremely cold winter climate. The animals cannot safely be allowed out of their housing for about seven months of the year, hence much of the mixing has to be done in a very restricted space. The animals are separately boxed and there is a natural reluctance on the part of the managers to mix animals which have not been in direct contact for a week or two. There have also been problems in the rhinos' diet, about which recommendations have been made by the CBSG deputation.

Moving the animals to a warmer climate would be the most desirable option but may not be realistic In view of political constraints. Adopting a two-year time limit for Improved breeding at Dvur Kralove, prior to suggesting a major translocation, 1a probably the best approach. The potential breeding animals are approximately 15 years old so they should theoretically have up to 15 years additional reproductive life. in the meanwhile, the Dvur Kralove staff must be given maximum encouragement and assistance with their efforts to build up this rhino group.

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REPRODUCTIVE RESEARCH UPDATE

Session Chairman BETSY DRESSER

INTRODUCTION

To start off this session, I would like to present the ideal scenario — the ideal for rhinos using the reproductive technology that has been hinted at during these meetings. That is, to collect semen for artificial insemination or embryos for embryo transfer, or better yet to be able to freeze semen and embryos and to move these cells around the country or around the world. We would like to bank these cells for years, thus helping to maintain an effective population size. That is the ideal. But the reality is that semen has been collected from black and white rhinos, it has been frozen and thawed successfully but it has never been used successfully to produce any offspring; artificial insemination procedures have been attempted in these species but have not yet succeeded. Embryos have not been collected from any species of rhinos nor, of course, have they been frozen. So we have a way to go.

Research is in its infancy and much of it needs to be applied, particularly the artificial reproduction techniques. in most cases — supplementing behavioural studies ——the greatest effort has focused on endocrine evaluations of oestrus cycles, and essentially we are still at the stage of trying to reliably determine the oestrus cycle of the rhinos in our care.

HORMONAL EVALUATIONS OF RHINO OESTRUS CYCLES AND PREGNANCY

A presentation was made by Dr. Ed Ramsay, formerly of the Oklahoma City Zoo, and Lonnie Kasman, formerly of the San Diego Zoo who, in a joint effort with Dr. Bill Lasley (also formerly of the San Diego Zoo) worked on a cooperative project with 19 zoos in North America.

With the forming of the AAZPA Species Survival Plans, around 1982, these researchers attempted to develop some strategies and techniques that the managers of rhinos in captivity might be able to utilize to help improve the captive breeding of their animals. The strategy that was adopted was to look at urinary steroid hormones; what was hoped was to better understand the reproductive physiology of the rhino (particularly the black and Indian rhinos) both through the oestrus cycle and pregnancy. Since blood is difficult to get from the animal when not immobilized, the strategy that has some obvious advantages is urine collection. In addition to being safer to collect, urine is readily available ln vast quantities!

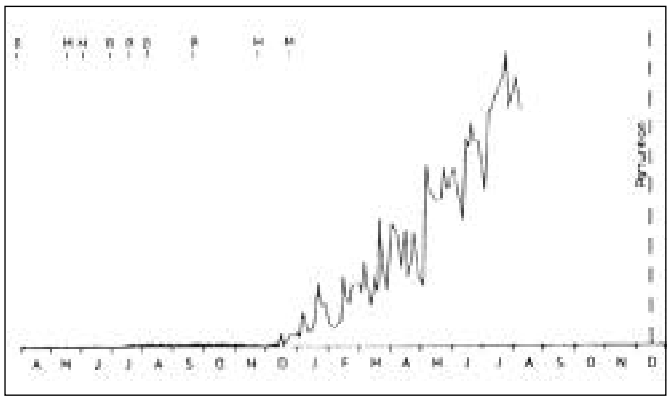
All of this work was done at the San Diego Zoo, and since the San Diego Zoo Endocrine Lab had a history of using radioimmunoassays for urinary steriod conjugate analysis, that is the method that was used. Preliminary studies there indicated that estrone sulphate, or estrone conjugates, would be useful for monitoring follicular activity in the Indian rhino and there was hope that it would also be useful in the black rhino.

Pregnanediol glucuronide (PdG) is an assay that was developed at the San Diego Zoo for monitoring luteal activity, or what was assumed to be luteal activity as a progesterone source in the rhino. That is the information that is presented in Figure 8.

The parturition which is indicated is the 1985 calf born at the Cincinnati Zoo. All the hormone values are indexed to creatinine to account for variability in the water content of the urine sample, so PdG is ng/mg creatinine.

Essentially what is seen are baseline levels (below the ensitivity of the assay) for about the first trimester of

Figure 8. PdG assay on one black rhino cow. B=breedings; 1 = mountings.



rpegnancy and then they begin to climb for approximately 12 months before parturition. The reason that the graph stops here is that Lonnie Kasman then left the San Diego Zoo and the assays ceased. This effort began in January 1983. At that time, there were only 27 black rhino females ln North America in the SSP programs. Of those, probably only about half were really considered to be potential breeders and then when the compliance factor was considered, there were actually only a few animals to study. However, this project shows the potential role that zoos can play in such research.

To further discuss urinary strategies, one thing that was hoped was to use the urine in the animals that consistently bred to diagnose pregnancy. There really has not been a good method in the past to monitor gestation or fetal viability. Then it was thought that it would be useful to look at the luteal phase, and ovarian function, in the case of pregnanediol luteal activity for comparison in the nonbreeding animal.

Unfortunately, pregnanediol was not found in the noncycling animal or the nonpregnant animal nor was it found in the first trimester of pregnancy in any of the black rhinos.

Figure 9 is a graphic representation of urinary pregnanediol ng/mg creatinine. The line on the right-hand side of the graph labelled zero indicates parturition during the course of the study and the bottom axis shows days prior to parturition. During the course of the study, eight animals that delivered were monitored and the graphed values represent samples from those eight animals. Unfortunately, the dots are not connected because frequently only half a dozen samples were received from an animal over the course of five or six months and to connect those dots would be deceiving. So this is merely a scattered representation of all the pregnancies that were looked at.

The one common factor is that around five months into gestation, between 12 and 9 months prior to parturition, a rise in pregnanediol was seen that continued throughout pregnancy in a rather consistent manner. So the serial sampling of three or four samples from an animal in late gestation can show measurable amounts of pregnanediol. It is believed that pregnancy in the animal can be determined. From data gathered during this project, it seems that the gestation range for black rhinos in captivity is 438-480 days, which is a range of 42 days with a mean of 463 days (a little more than *15* months). There is also an indication that post conception breeding occurs in these animals and can confuse rhino managers.

Figure 10 represents data obtained from Lottie from the Oklahoma City Zoo during a pregnancy. The scale on the left hand side is estrone sulfate or estrone conjugate and the scale on the right is pregnanediol glucuronide. The levels rise just into the measurable range (which ln this assay was about 8 ng/mg creatinine) about one year prior to parturition. The other thing that this figure shows is that there is a very precipitous drop-off in pregnanediol prior to parturition.

The project initially involved the black and Indian rhinos. in the second year, however, a few urine samples from two noncycling white rhinos were included in the assays. Non–cycling means that these are animals that were not being bred, were not showing any external signs of estrus and (as with the black rhino) had no measurable pregnanediol. Samples from two pregnant white rhinos were also collected, and levels of pregnanediol were found in late gestation that compared closely with those of the black rhinos.

In the Indian rhino it was possible to characterise both follicular and luteal phases and estrone conjugate and pregnanediol were found to be very useful for looking at both the estrus cycle and pregnancy. The Indian rhino is remarkably different to the black rhino, excreting a far higher level of steroids; during pregnancy the pregnanediol levels in the Indian rhino begin to climb at a similar time (about the beginning off the second trimester) but go up into the microgram/mi creatinine range.

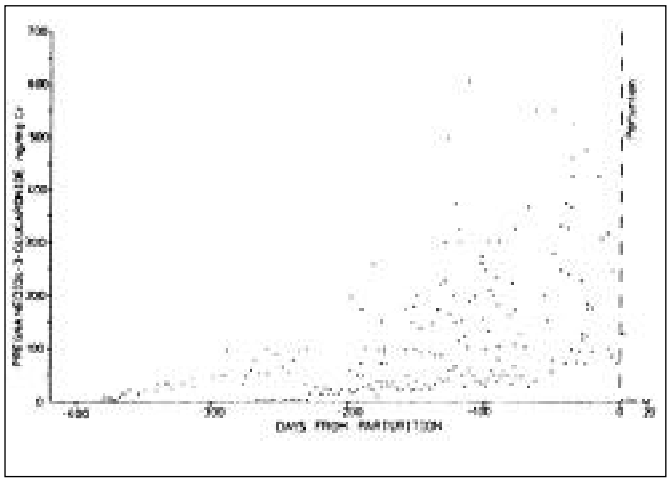


Figure 9. Urinary PdG mg/mg creatinine from 8 black rhino cows.

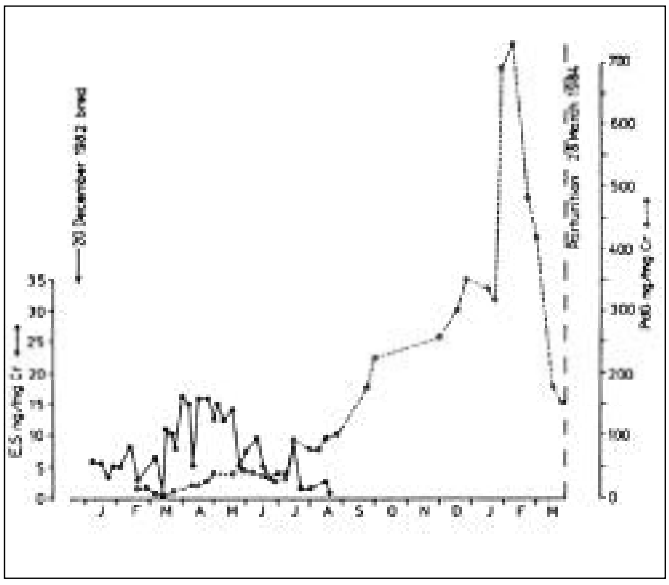


Figure 10. Estrone sulphate and PdG assays on one black rhino cow.

A cyclic pattern was evident ln estrogen values measured in the two Indian rhinos; at the end of a follicular episode, estrone sulfate levels dropped and then some 40 days later rose again, stayed up for 7-10 days and thereafter declined again. When estrogens declined, pregnanediol levels increased, indicating the production of a corpus luteum (which secretes progesterone). Some 14 days later the pregnanediol dropped again. These measurements tied in with behavioral indications of estrus ln these animals.

ESTRUS CYCLE DETERMINATION FROM CONDITION OF REPRODUCTIVE TRACT

Dr. Robert Wagner, veterinarian at the Pittsburgh Zoo. collected data from an 8 year old female southern white rhino over a period of 20 months. Attempts were made to collect samples biweekly. Behavioural observations for stage of estrus cycle were correlated with:

1. Rectal examination for uterine and cervical tone
2. Vaginal cytology
3. Urine hormones:
   (a) total estrogens (estradiol-17-B, estrone and estrone sulphate)
   (b) progesterone

Rectal examination of uterus and cervix revealed much Information about the female's cycle and anatomy. Ovaries were not palpable. The reproductive tract tone showed a change from being soft, pliable and flaccid to becoming firmer for some weeks prior to behavioural estrus, and then rapidly became well defined and turgid for two to four days during behavioural estrus.

Vaginal cytology was reported by Spellmore and Booth in AAZPA Regional Proceedings in 1981 for a black rhino. Similar findings were seen in the cytology of the white rhino. During diestrus, round non-cornified epithelial cells with distinct nuclei were seen, along with small quantities of mucus and debris. Then for about two or three days during proestrus the epithelial cells cornified and became angular ln shape with pyknotic and darker nuclei. A slight increase in mucus and debris was noted at this point. Also at this time the cells began karyolysis and lost their nuclei. A sudden change at estrus in the non-cornified to cornified cell ratio (NC/C) often occurred within 12 hours; commonly, greater than 70% of the cells became cornified with considerable debris noted. The epithelial cells of estrus were then irregular in shape with edges folded over, and contained no nuclei. The NC/C ratio would revert back within 12 hours to 50/SO or greater with cells resembling new diestrus cells. Rapidly changing cytology seen ln Pittsburgh's white rhino closely agrees with reports from San Diego Wild Animal Park of estrus lasting 15 hours based on behavioural observations (1985 SSP Survey).

Hormone analysis of urine for total estrogens and progesterones was completed as frequently as possible but occasionally time gaps of up to 12 days since collection would occur. Analysis was done by radioimmunoassay (RIA). Hormone concentrations were corrected for dilution by standardizing against creatinine levels. Baseline estrogen levels ranged from 200 to 900 pg/ml with small mid-cycle peaks of less than 900 pg/ml ranging between November and July. Total estrogens showed the best correlation with observable heat. Estrogen peaks of greater than 1 200 pg/ml occurred within four days of noted heat. From August to October multiple estrogenic peaks (less than 1 300 pg/ml) were seen with little pattern or regularity. During this time. poorly defined heats or no cyclic behavioural activity was seen. Progesterone peaks (0.12S-0.250 ng/ml) followed extremely close to declining estrogen peaks from November to July, then levels became erratic and poorly correlated. These hormone fluctuations may explain the lack of obvious estrus behaviour in Pittsburgh's female rhino from late summer to early winter. There seems to be a seasonal anestrus occurring in this female during this time.

From December to July, Pittsburgh's female has strong (easily observed) heats and regular estrus cycles. With approaching heat the uterus and cervix increase tone, the vaginal cytology changes from non-cornified to cornified cells and urine total estrogen levels peak. Behavioural estrus lasts three to five days. Progesterones rise after estrogen peaks and tone and vaginal cytology go back to baseline levels. Cycle length varies from 38 to 58 days with most cycles being 40 to 42 days. As mid-summer approaches, cyclic behaviour and observable heats are much harder to determine. This agrees with the non-cyclic activity in tract tone and cytology. Future goals are to isolate a LH-like compound in the urine, sonographic evaluation of ovaries for staging the cycle and eventually artificial insemination.

FURTHER RESEARCH ON METHODS FOR OVULATION AND PREGNANCY DETECTION

Dr. Richard Kock of the London Zoological Society presented results of studies done in collaboration with Dr. J.K. Hodges also of the London Zoo, on detection of ovulation and pregnancy in rhinos. The following is a summary of the results.

1. Comparison of urinary estrogen metaboiltes during pregnancy. Sequential hydrolysis of urine samples from mid-late pregnancy in the Indian, black and white rhino showed:
   (a) important species differences in the amounts and type of estrogen excreted;
   (b) large amounts of estrogens were detected during pregnancy in the Indian species. The most abundant estrogen component was estrone sulfate;
   (C) very low levels of estrogen were excreted in urine during pregnancy ln the black and white rhinos. Of those measured estradiol glucuronide appeared to predominate;
   (d) measurement of urinary estrogens may be useful for monitoring pregnancy in the Indian rhino but not at present in the other two species;
   (e) more studies are needed in the black and white rhinos to examine the presence of other urinary estrogens and to determine whether there is a preferential route of fecal excretion.
2. Measurement of urinary progesterone metabolites during pregnancy.
   Urinary pregnanediol-3x-glucuronide was measured during mid-late pregnancy in the Indian, black and white rhinos. The results showed:
   (a) elevated levels of PdG in all three species;
   (b) levels ln the Indian rhino were between 5-10 ug/mg creatinine whereas levels in the black and white rhinos were much lower at comparable stages of pregnancy (0.4-0.8 ug/mg Cr and 0.05-0.1 ug/mg Cr, respectively);
   (c) levels of PdG in all three species fell markedly (greater than ten-fold) within one week of termination of pregnancy (birth or abortion);

(d) measurement of urinary PdG appears to provide a useful method for detecting mid-late pregnancy in rhinos. Further work is needed to establish tests for early pregnancy.

3. Monitoring of estrus cycles and ovulation.
   In contrast to the Indian rhino, attempts to monitor the estrus cycle in black and white rhinos by measurement of urinary estrogen metaboiltes and pregnanediol-3x-glucuronide have so far proved unsuccessful. Other methods need to be investigated.
4. New assay methodology.
   A new, simple microtitre plate ELISA (enzyme assay) for urinary pregnanediol-3x-glucuronide has been developed and validated for all three species of rhino.

UPDATE ON DEVELOPMENT AND APPLICATION OF REPRODUCTIVE TECHNOLOGY TO RHINOS

As head of the research team of the Cincinnati Wildlife Research Federation (CWRF) which is a combined effort of the Cincinnati Zoo, Kings island Wild Animal Habitat and the University of Cincinnati College of Medicine, Dr. Betsy Dresser reported on the development and application of reproductive technologies to rhinos. At the Cincinnati Zoo , there are two breeding pairs of black rhinos and they have produced 13 offspring to date. At Kings Island, there is a group of white rhinos, and four nonpregnant cows that are being worked with now in some areas of embryo transfer technology.

It is the hope of the CWRF to eventually be able to do embryo transfer within the white and black rhino species and also at some point attempt interspecies embryo transfer between the black and white rhinos. There is a lot of talk about embryo transfer but until actual manipulation of these animals is tried, working with them is a little more difficult than is first thought. So, first there is a need to determine if catheters can be physically inserted into the cervix and uterus manipulated before superovulation by hormones can be attempted.

As has been mentioned by other investigators, there is a need to be able to determine the estrus cycle in rhinos, It will be important to know when we can artificially inseminate on when to breed these animals before embryo transfer can be attempted. And then, after that, we have to know when embryos can be recovered. Also, embryo recipients will need to be hormonally prepared in order to establish a pregnancy. At Kings Island in Ohio, in an ongoing effort to develop embryo transfer technology for white rhinos, animals were first immobilized, placed in sternal recumbancy and rectally palpated to evaluate the reproductive tract. To date, it has been determined that uterus and ovaries could be palpated. but it is often very difficult. Ultrasound equipment is now being used to aid these efforts.

Specula are being developed in order to visualize the cervix for catheter insertion. A lengthy catheter has been developed for this procedure and attempts to flush the uterus with fluids are underway. Once superovulation techniques are pursued. embryo recovery techniques will be correlated.

Another technique that the CWRF team have been trying with rhinos came out of work that is being done with domestic cattle. It involves a small radio transmitter that sends out pulses. It has been used successfully in cattle to determine internal body temperature. It is inserted into the vagina and is similar to the method used to measure internal body temperature in women when they ovulate and there is a measurable increase in body temperature. When a cow's internal body temperature increases, these pulses increase and are received through a radio receiver. Dr. David Zartman, of Ohio State University. has inserted many of these into cattle. He custom-made the transmitter for the rhinos (larger that that used in a horse). The rhino cows have been monitored for at least six months and a trend does appear to be emerging.

Dr. Terry Blasdel. research coordinator for the Houston Zoo, has organized a program to produce offspring from white rhinos at the Houston Zoo by artificial Insemination. This project involves at least eight other zoos in North America, but had not yet begun at the time of the meeting.

Session Chairman ERIC MILLER

HEMOLYTIC ANEMIA IN THE BLACK RHINO

Summary of presentation by R. Eric Miller (St. Louis Zoological Park), co-authored by Hugh Chap//n (Washington University School of Medicine), Donald E. Paglia (University of Cal/torn/a at Los Angeles) and Will/am J. Boever (St. Louis Zoological Park).

Hemolytic anemia in the black rhino *(Diceros bicornis)* is a frequent occurrence and cause of death in the captive population of this species. Twenty-eight episodes of hemolytic anemia have been identified in 21 animals in zoos in North America, Europe and Japan. Eighty percent of the affected rhinoceroses have died during their initial or a recurrent episode of the anemia.

In man and in domestic animals, hemolytic anemia may result from a variety of factors that lead to a decrease in the survival time of the red blood cells (RBC's) and their early intra- or extra-vascular destruction within the body. Intravascular destruction of the RBC's leads to the release of their hemoglobin into the serum (hemoglobinemia) and may result in its passage into the urine (hemoglobinuria). The latter results in a clear, dark red coloration in the urine that is often the first sign that a black rhinoceros is developing a hemolytic crisis.

The case that occurred in St. Louis in 1981 (studbook 183/STL 6) was typical of the majority of the cases (8). A nine-year-old nulliparious female was noted to be weak and passing red urine. She was anesthetized for further evaluation, and blood values reflected a marked anemia — a haemocrit of 14.5% (normally 45-50%) (6). in other cases this value has ranged from 4.5% to 36**%** on initial presentation. Nucleated red blood cells — cells that in the horse are indicative of intensive efforts to replace the RBC loss—— were noted Similar findings, including regenerative bone marrow, have been found in two subsequent cases. The St. Louis animal died during attempts to reverse the anesthetic, no doubt complicated by the severe anemia present. Necropsy find-