An Analysis on Cyclicity and Pregnancy in the Southern White Rhinoceros (*Ceratotherium simum simum*) by Noninvasive Progesterone Monitoring using VHF Radio telemetry

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Three female free-ranging white rhinoceroses in South Africa were monitored noninvasively and with the use of VHF radio telemetry by collecting fecal samples for progesterone metabolites measurement. All animals were immobilized so that radio transponders could be added in their horns. During this procedure blood samples were also collected. Blood assay results indicated pregnancy at the time of immobilization in two of the three rhinoceroses. Fecal samples were collected for a period of 90 days at about weekly intervals. Fecal sample collection from one rhinoceros failed, due to behavioural factors. Progesterone profiles of two rhinoceroses were created in this study. Concentrations above luteal phase values were found for both rhinoceroses during pregnancy. During the 50 days prior to parturition fecal progestagens declined, a feature so far only described in black rhinoceroses. Within 9-12 days *post partum* the progesterone concentration had reached follicular phase values. It was concluded that measurement of progestagens in feces with Enzyme Immunoassay (EIA) enables noninvasive monitoring of pregnancy (and presumably also cyclicity) in the southern white rhinoceros. Collectively the information generated in this study contributes to a better understanding of monitoring reproductive endocrinology in wild white rhinoceroses, information invaluable to the conservation and management of this species. This study also tried to emphasize the importance of further research on wild rhinoceroses to help the efforts to conserve this species on the long term.

Keywords: *Ceratotherium simum*, fecal progesterone metabolites, pregnancy, VHF radio telemetry, RFID technology, rhinoceros

1. Introduction

The African white rhinoceros (*Ceratotherium simum*), which has barely been rescued from extinction at the end of the 19th century, is one of the five species of rhinoceroses alive today. Two genetically distinct subspecies exist, namely the northern white rhinoceros (*Ceratotherium simum cottoni)* and the southern white rhinoceros *(Ceratotherium simum simum).* Together with the other surviving rhinoceros species: black (*D. bicornis*), Indian (*R. unicornis*), Javan (*R. sondaicus)* and Sumatran (*D. sumatrensis*), the white rhinoceros population is looking at an uncertain future [Estes, 1991]. One of the major causes is the demand for its horn, which is being used as an ingredient in traditional medicine and also in the making of ceremonial curved daggers in the Middle East [Owen-Smith, 1992]. Horn poaching involves killing the animal and cutting off its horns. The demand for rhinoceros horn in the world is still very high and finding strategies to fight against the poaching plays a key role in maintaining the rhinoceros populations today. Also habitat loss and human disturbance play a role. The southern white rhinoceros is the most numerous of all, with an estimated population of 17,480 [Emslie et al., 2007; IUCN, 2008] of which 94% live in South Africa. Although this number seems viable, rapid decimation of the population, as observed in other rhinoceros species, should be avoided. Being classified as "Near-Threatened" on the IUCN's Red List of Threatened Species, the southern white rhinoceros population is subject to attention and highly dependent on effective protection and intensive conservation and management [Amin et al., 2003; Hermes et al., 2005].

Figure 1 – A southern white rhinoceros adopting a rump-to-rump posture of alertness, by pressing herself against her calf [Owen-Smith, 1992].

Breeding is an important component of rhinoceros conservation, with the captive populations also serving as potential reservoirs for reintroduction into the wild. However, reproduction in white rhinoceroses – especially those in captivity – has been distinctly

disappointing [Patton et al., 1999; Swaisgood et al., 2006]. Reasons for this are still unclear. Our understanding of the reproductive biology of the southern white rhinoceros is still limited and details are largely unknown. Only few observations have been made related to reproduction in the wild. Bertschinger, who wrote a review on the more important literature on the reproduction of black and white rhinoceroses, found an average estrous cycle length of 28 days in white rhinoceroses in the wild [Bertschinger, 1994]. Other literature reports the existence of two different cycle lengths in captive white rhinoceros, a shorter (30-35days) and a longer (65-70days) cycle, with only the shorter cycle being a fertile cycle [Patton et al., 1999; Hermes et al., 2007; Schwarzenberger et al., 1998; Morrow et al., 2008]. It appears that wild white rhinoceroses only demonstrate the shorter cycle [MacDonald et al., 2008]. Also, periods of acyclicity have been found with a high incidence in white rhinoceroses in captivity [Brown et al., 2001]. Hermes and Hildebrand et al. suggested that this is a consequence of long non-reproductive periods [Hermes et al., 2006]. Additional research is necessary to further understand the reproductive performance of this species.

Measuring formed metabolites of the reproductive hormone progesterone can be very useful for monitoring reproductive physiology. Progesterone drives the reproductive processes in female rhinoceroses and reflects the ovarian function. With the development of fecal hormone assays, it is now possible to collect samples in a non-invasive way. Apart from the practical advantages, this technique also bypasses the negative effects of stress on the results when using invasive methods (involving venipuncture) [Christensen et al., 2006; Wittemyer et al., 2007]. Once the progesterone level of free-ranging females can be determined intensively on a regular basis and over a long period, this could increase the knowledge on the reproductive status of the female white rhinoceros in its natural environment.

Previous studies showed that localizing free-ranging rhinoceroses for sample collection is difficult, which might also be a contributing factor in the lack of data [Strike and Pickard, 2000]. The use of VHF radio telemetry, where small transponders are implanted either subdermally or in the horns of animals, may provide an easy and effective monitoring tool. If effective, it will make regular sample collection better possible.

The aim of the current study is to develop a monitoring system for measuring fecal progesterone metabolites in free-ranging white rhinoceroses, and use this approach to better understand cyclicity and pregnancy in the white rhinoceros, which subsequently might be used to help the efforts to conserve this captivating species in the long term.

2. MATERIALS AND METHODS

Animals and study area

The Lapalala Wilderness is one of the privately owned game reserves in the Limpopo province of South Africa where the southern white rhinoceros as well as the black rhinoceros have been successfully introduced. The white rhinoceros population using this reserve consists of approximately 39 individuals (black rhinoceroses approximately 21). Rainfall averages an estimated 500 mm per year with a mid summer (January-February) seasonality, and the rhinoceroses are being fed additionally from May/June till Oct/Nov. This 36,000 ha enclosed reserve provides a sanctuary for the breeding of endangered animals, and forms a good area for reproductive performance investigation in rhinoceroses [Walker, 1994].

Three fertile, middle aged, female southern white rhinoceroses, with calf and with different

Study animal	Age (yrs)	Age calf (yrs)
Radimpe	**13.8**	**2.9**
Motklaki	**15.2**	**1.9**
Honkey	**26.1**	**4.9**

Table 1 – Summary of white rhinoceroses used in this study.

home ranges have been accurately identified for this study, by gathering documented data on the behaviour and home range (sizes) of population

Figure 2 – Addition of radio transponder. A team of veterinarians drilling a hole in largest horn of immobilized Motklaki.

individuals. Our preference went out to animals with different home ranges, so that larger variability was implanted, but also in order to make the radio telemetry testing more reliable. Subsequently we looked at calf ages, in order to increase the chance on fertile, cyclic individuals (Table 1). In each rhinoceros a transponder was implanted in its largest horn (Fig. 2). During this immobilization animals were examined thoroughly, the body temperature was measured, blood serum and rectal feces samples were collected, and ear notches, each with a unique pattern, were made. Serum has been tested for pregnancy by a direct, quantitative progesterone measurement, using a Coat-a-count Progesterone solid phase Iodine 125 radio immunoassay.

A parallel study, but without the use of VHF radio telemetry, has been executed at the same time in the same reserve, for which three other female white rhinoceroses have been identified. Materials and methods in this study have been implemented equally to this study and its results can therefore be used as a comparison model.

Sample collection

Each rhinoceros was tracked and observed at least once a week, during the first or last 3 hours of daylight (starting at respectively 5:45h and 14:00h). Documented information available in the reserve defined the home ranges of each rhinoceros. By car we went to a starting point (often the place where feeding takes place during the dry season, rhinoceroses tend to remain close to where the food is deposited for them). From there, tracking would take place. For which we often had to leave the car and proceed by foot. Identification followed a combination of VHF radio telemetry (Communications Specialists 148/152 MHz manufactured by Africa Wildlife Tracking cc, Rietondale, Gauteng, South Africa), on foot track-tracing, ear-notch identification and other physical characteristics of the rhinoceros. Also a

Figure 3 – VHF radio telemetry (left) and footprint tracking (right), two methods used in this study for tracing rhinoceroses.

combination of characteristics of the calf, like ear-notch identification and body size, were used. Documentation was also made on group

members seen together with our study animals. This could give information on group behaviour and could therefore be an aid in finding rhinoceroses in the future, and might also be of value in future processes of identifying new sample animals.

In order to prevent disturbance of the study animals and other group members we had to take into account wind directions and noise prevention. Individual fecal samples (10-40g) were collected fresh from the ground directly

Figure 4 – Collecting a fresh fecal sample from Radimpe (also on picture).

after observation of defecation (Fig. 2), one or two times per week, or sporadically without observation within 30 min after defecation. This was documented immediately. For this we used hand gloves (Hartmann Peha-soft, REF:942150). Schwarzenberger found that progesterone metabolite concentration within the feces does not differ significantly between the central portion and the outer layer of the fecal ball [Schwarzenberger et al., 1998]. Balls were chosen that were still whole and clean of insects (dung beetles arrive rapidly after defecation). Subsequently we opened one or more balls and collected small parts of the inside of different balls, avoiding and removing undigested material.

Samples were also judged on consistency, color, smell and the presence of parasites. If a parasite was present (n>1) it was collected and preserved in 10% formalin, as an additional indicator of the general health of each individual. After collection into glass sample cups, samples were temporarily stored in a cooler box and then stored frozen at -20°C until analysis.

One of the rhinoceroses, Honkey, appeared to be very difficult to trace, due to her behaviour. Her home range was in an area where access was difficult and she and her calf were very alert and fled without stopping whenever we tried to get her in sight. Therefore no samples have been taken

from this individual, apart from the rectal sample taken during the immobilization. From Radimpe and Motklaki we collected an average of one fecal sample per animal per week. This study was carried out from October 2008 to January 2009.

In order to determine whether the use of VHF radio telemetry really improves the research, time was documented each time we searched for a rhinoceros at the starting point of tracking and on the moment the rhinoceros was found. This was also done for three rhinoceroses that we regularly tracked for a parallel study, but without the use of radio telemetry.

In order to avoid the risks involved while executing this project it was essential to respect safety rules as laid down by the Lapalala management. Because of the aggressive and territorial nature of the white rhinoceros (and especially the black rhinoceros, which could also be encountered) it was important to be concentrated and aware of possible danger at all times [Owen-Smith, 1992]. Other precautions taken were: respecting speed limits, being in the company of an experienced guide, never walking alone and no splitting up of the group, avoidance of any kind of disturbance of animals, keeping a considerable distance from the rhinoceroses and strictly no talking when walking through the bush.

Fecal extraction

In order to get the progesterone metabolites out of the sample material and into a liquid solution suitable for the fecal hormone assay, the fecal samples had to be extracted. Methods are used as described by the 'Standard Operating Procedure – Extraction Method for Dry Fecal Samples' of the Onderstepoort Veterinary Institute in Pretoria (Appendix A). Prior to extraction, the samples were freeze-dried in a vacuum oven (Instruvac Freeze-drier from Air & Vacuum Technologies, Pretoria, South Africa, Model: VFDT 02.50) for 28.5 hrs at -85 kPa to reduce variability in water content.

The first step in extraction was the pulverization of the freeze-dried material. For this, the entire cup content was removed using tweezers and placed into a sieve. Subsequently, the matter was scratched around in the sieve, allowing only fine powder to fall through. This powdered matter was then placed back into the cup. To avoid cross-contamination, the tweezers and sieve were soaked in 80% EtOH (prepared from Ethanol Absolute 99%, Merck, Saarchem, diluted with distilled water) between each sample. Hand gloves were also changed between samples and the working surface was

kept clean. After this step samples were stored at room temperature. In the next step 0.05g (0.050-0.055g) of each sample was weighed and put over into a sample tube (Kimble Borosilicate Glass, Disposable Culture Tubes, 12x75mm). The actual weight was recorded for each individual sample. For the final separation 3ml of freshly prepared 80% EtOH was added to each tube. All samples were placed onto a multi-shaker on high speed for 15 minutes and thereafter centrifuged for 10 minutes on 3000 rpm. With a pipette 1.5 to 2ml clear supernatant was removed from each tube and transferred to a fresh tube. These tubes were stored upright at -20 °C, ready for the Enzyme Immunoassay (EIA).
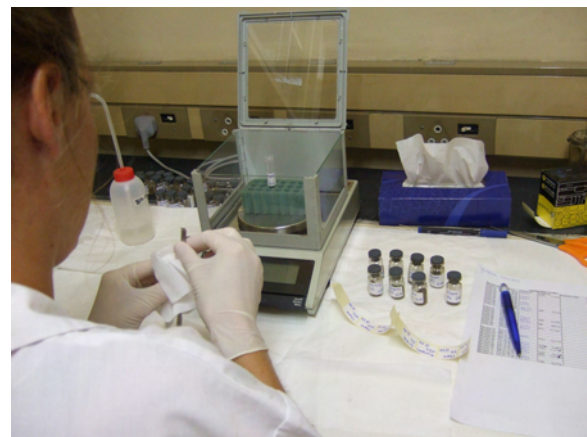
Figure 5 – Pulverization of freeze-dried material (above) and weighing of material (below) in the process of fecal extraction.

Assay procedures

For the EIA, the 'Standard Operating Procedure: Enzyme Immunoassay On Microtitre Plates Using Biotinylated Steroids As Labels' (Appendix B) was followed.

Dilutions (1:200– 1:1000) in assay buffer (Sodium chloride at 0.145M, Disodium phosphate at 0.042M, and Bovine Serum Albumin [BSA] at 0.1%, pH 7.2) were made of

the extracted material in preparation to the progesterone EIA. The EIA relied on a monoclonal antibody raised against 5beta-pregnan-3alpha-ol-20-one. Progesterone standard (4-pregnene-3,20-Dione) and biotinylated labels (5alpha-pregnane-3beta-ol-20one 3HS:DA DOO-B) were used. The sensitivity of the assay determined at 90% B/Bo was 0.3 pg per well. Inter- and intra-assay coefficients of variance ranged between 6.4% and 10.5%. The immunoreactive progesterone concentrations are expressed as ng per g dry weight of rhinoceros feces. The assay has been validated for rhinoceros feces and samples were run in duplicate. The optical density was measured for its absorbance at 492 nm, using an 8-channel microplate reader (Labsystems iEMS MF).

Data analysis

Reproduction status profiles of the study animals were evaluated on indications for cyclicity or pregnancy. Mean concentrations of progesterone metabolite (pregnane) were calculated for both pregnant rhinoceroses. A two-sample *t*-test assuming unequal variances was used to compare Radimpe's mean pregnane concentration prior to parturition with the mean concentration post partum. A linear mixed-effects model was used to determine whether pregnane concentrations in fecal extracts from the pregnant rhinoceroses rose or declined significantly during pregnancy and/or prior to parturition.

3. RESULTS

All fecal samples collected were analyzed with the EIA (Appendix C). Pregnancy tests of the blood samples of all three rhinoceroses indicated pregnancy in Radimpe and Motklaki,

but not in Honkey (Table 2). However, false positive results can occur in the presence of pyometra and false negative results can occur during early pregnancy (< 4 months) and late pregnancy (last 2-4 weeks) [Strike and Pickard, 2000]. The predictive value of the Coat-a-count Progesterone solid phase Iodine 125 RIA used is very good from 4 to 16 months pregnancy. From Honkey only a rectal fecal sample was collected on October 21, 2008. The pregnane concentration for this sample was 7.1 ng/g

| Animal	Prog (nmol/l)	Pregnancy
Honkey	0.12	**Non**
Motklaki	57.00	**9-12 months**
Radimpe	103.89	**13-16 months**

Table 2 – Serum [prog] assay results - pregnancy status. Non pregnant = Less than 4.5 nmol/l, 9-12 months pregnant = 50-70 nmol/l, 13-16 months pregnant (white rhino) = 70-90 (and greater) nmol/l. Sample collection dates were October 21, 2008 for Honkey and Motklaki and September 2008 for Radimpe [Veterinary Research Institute, Pretoria].

feces. This is in line with the pregnancy test result of Honkey. Because both Radimpe and Motklaki appeared to be pregnant, pregnane levels for these females did not indicate luteal phases (Fig. 7a and b). On December 10, 2008 Radimpe delivered a live, male calf.

The mean fecal pregnane concentration was 273.3 ± 97.8 ng/g feces for Motklaki. In Radimpe, the mean pregnane concentration, during the 50 days prior to parturition, was 412.4 ± 144.2 ng/g feces. After parturition, Radimpe showed a mean concentration of 25.8 ± 26.7 ng/g feces, significantly different to her pregnant concentration mean (*t*-test: two-sample assuming unequal variances, $p<0.05$, N=5, N=6). The concentration declined to follicular phase values within 9 -12 days post partum and remained in this range for the following month. Linear mixed-effects modelling with random animal effects and time and animals and their interaction as fixed effects, showed a significant

Figure 6 – Dispensing stop reagent H_2SO_4 into each well (left). The blue colour turns yellow (right).
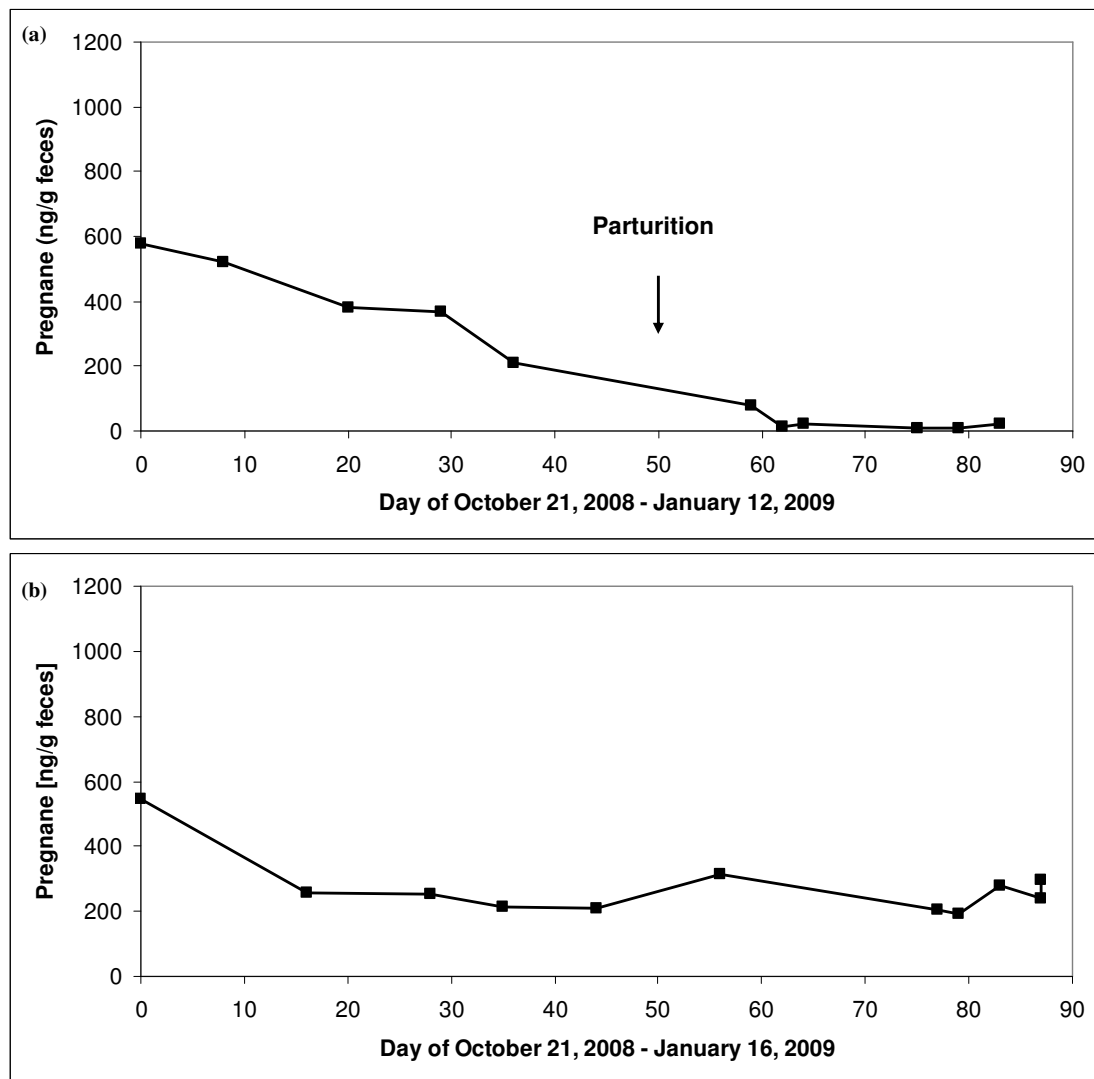
Figure 7 – Concentrations of fecal progesterone metabolite (pregnane) in southern white rhinoceros. **a:** Radimpe, October 2008-January 2009, the arrow (↓) indicates observed moment of parturition. **b:** Motklaki, October 2008-January 2009.

decline of pregnane concentration prior to parturition in Radimpe (regression coefficient = -8.16 with standard error 2.655, t = -3.074, p = 0.0106). Motklaki's profile showed a significant deviation to Radimpe (difference in regression coefficients is 5.89 with standard error 2.702, t = 2.178, p = 0.0521), which means no significant decline was present in Motklaki. In this study, proving the benefits of VHF radio telemetry objectively was very difficult. The reason for this was the variation in behaviour found for animals in both study groups. Nevertheless, during the research one could generally see that using telemetry indeed had a positive effect on the speed of finding rhinoceroses.

On 03-12-2008, however, problems started to evolve around the functionality of the system. Radimpe's transponder started to dysfunction slowly until no signal was received at all. This took place within 5 days. On 22-12-2008 Motklaki's transponder stopped working as well.

4. DISCUSSION

Fecal samples of white rhinoceroses were used to test the possibility of monitoring cyclicity and pregnancy by non-invasive progesterone investigations in this species. Intercalving period is given as 1.5–3.45 years [Owen-Smith, 1992; Bertschinger, 1994; Hildebrandt et al., 2007]. Patton et al. described

an estimation of the onset of a luteal phase to occur 6 days before the first pregnane value rises above 150 ng/g feces. Concentrations above 150 ng/g feces were observed for the entire period in Motklaki (Fig. 7b). Radimpe showed concentrations >150 ng/g feces until day 36 (prior to parturition). The first sample after the moment of parturition, collected on day 59, gave a pregnane concentration value <150 ng/g feces (Fig. 7a). Patton et al. also found that pregnane concentrations during pregnancy surpass non-pregnant luteal phase concentrations [Patton et al., 1999].

Schwarzenberger et al. found a decline in fecal progestagens during the last two weeks of pregnancy in the black rhinoceros [Schwarzenberger et al., 1993]. Patton et al. did not found this decline in the white rhinoceros [Patton et al., 1999]. In this study however, a significant decline prior to parturition was present in one of the study animals. To further invest this, more research is necessary, using more study animals.

When considering the addition of animals to this ongoing project in the future, gained information in this study on group behaviour can be used. The current study showed that particular individuals, namely Pedi (current age 16.2 years) and Mokibelo (current age 5.4 years, not sexually mature yet), are physically and practically interesting animals for future developments.

Another facet during this study was investigating the use and efficiency of VHF radio telemetry. Because of the large variation in general behaviour of the six used study animals it was hard to give significant conclusions based on the documented time-data. One animal without transponder was hand-raised and because of that easy to find in general, standing often beside the road and not fleeing for human. While one of the animals with transponder was extra hard to find due to the hiding behaviour she adopted around parturition. However, in general terms the technique definitely contributed to the speed of finding those three rhinoceroses, for as long as the system was functioning properly. The device gave information on the direction of the rhinoceros, but also on the distance to the rhinoceros. And taking this information into account made it possible to better adjust our searching strategies (driving and walking speed adjustment, keeping larger distances because in-

sight identification was less necessary). At the point that Radimpe's transponder stopped functioning, Motklaki's and Honkey's transponders were still sending signals to the receiver, indicating that the problem was lying in the transponder or in the rhinoceros(horn) presence and not in the receiver. Further research is being executed to find the causes for the fact that two transponders stopped functioning. A described cause is damage due to the combination of horn growth and horn wear [Toit, 1996]. This taking place makes it reasonable to search for better functioning technologies. A practical potential solution for making accurate monitoring possible is the use of a Radio Frequency Identification (RFID) system to track rhinoceroses. RFID is a proven electromagnetic technology for identifying (moving) objects [Hahnel et al., 2004; Roberts, 2006]. The technology has also been used in field research to trail fish, showing its migration patterns [Skov et al., 2008]. One advantage of this technology is that the transmitter is very small, which makes it possible to inject without immobilization and less sensitive to destruction. Secondly, more accurate positional data can be retrieved without the need of using an antenna in the field (data will be sent to a stationary computer). Further research needs to be done to determine the possibilities and advantages of using RFID as a supporting technology to improve noninvasive monitoring in future reproduction research of rhinoceroses.

5. CONCLUSION

Aim of this study was to develop a monitoring system for measuring fecal progesterone metabolites in free-ranging white rhinoceroses. In the past, little research has been done on wild rhinoceroses, compared to zoo animals, because of the practical difficulties involved. This study developed a functional method in order to produce (cyclicity and) pregnancy profiles of free-ranging rhinoceroses. Profiles of two pregnant rhinoceroses have been created within a time span of approximately 90 days. The results of present study indicate that non-invasive monitoring of rhinoceroses can be used to create reproductive status profiles of wild rhinoceroses, and that the use of VHF radio telemetry (and in the future perhaps RFID Technology) can be a valuable tool. However, because of the short time period, only a little

amount of data could be collected per study animal, and it is necessary to make this study and studies equivalent to this one, longitudinal studies with the implementation of more study animals and control animals.

6. ACKNOWLEDGEMENTS

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APPENDIX A

STANDARD OPERATING PROCEDURE

EXTRACTION METHOD FOR FAECAL SAMPLES
University of Pretoria, South Africa

1. 0.5 g wet faecal sample.
2. Add 1 mℓ dH$_2$O.
3. Add 4 mℓ Methanol.
4. Shake for 30 minutes at room temperature.
5. Centrifuge for 15 minutes at 3700 rpm at 5°C.
6. Take off 1 mℓ of the supernatant.
7. Add 5 mℓ Ethyl Ether.
8. Add 250 mℓ of a 5 % NaHCO$_3$ solution in dH$_2$O.
9. Vortex.
10. Centrifuge for 15 minutes at 3700 rpm at 5 °C.
11. Freeze (overnight at –20 °C or 30 minutes at –70 °C).
12. Pour off supernatant.
13. Dry under N$_2$ at 45°C.
14. Add in 500 µℓ assay buffer (buffer 1.3.) to dried tube.
15. Vortex and let stand for 20 minutes. *Take note:* The whole pellet does not dissolve.
16. Dilute samples in assay buffer (buffer 1.3.) if required.

STANDARD OPERATING PROCEDURE

ENZYME IMMUNOASSAY ON MICROTITRE PLATES
USING BIOTINYLATED STEROIDS AS LABELS
Dr. Andre Ganswindt, University of Pretoria, South Africa

1.   Buffers:

1.1.   **Coating Buffer:** *(use immediately & discard unused buffer)*
1.59g Na_2CO_3
2.93g $NaHCO_3$
Add 1ℓ dH_2O
Adjust pH to 9.6
(Add 300 µl per well containing 1ug Coating IgG for each well)

1.2.   **Second Coating Buffer (Saturate solution):** *(use immediately & discard unused buffer)*
8.5g NaCl
3g BSA
5.96g Na_2HPO_4
Add 1ℓ dH_2O
Adjust pH to 7.2

1.3.   **Assay Buffer:** *(store up to 4 weeks at 4 ℃)*
8.5g NaCl
1g BSA
5.96g Na_2HPO_4
Add 1ℓ dH_2O
Adjust pH to 7.2

1.4.   **PBS-Solution:** *(store up to 4 weeks at 4 ℃)*
0.136mol NaCl (7.94g)
8.1mmol Na_2HPO_4 (114.98g)
2.7mmol KCl (20.13g)
1.5mmol KH_2PO_4 (20.41g)
Add 1ℓ dH_2O
Adjust pH to 7.2

1.5.   **Wash Solution:** *(store up to 4 weeks at 4 ℃)*
9.6ℓ dH_2O
0.05% Tween 20 (0.5 mℓ)
400mℓ PBS-solution

1.6.   **Stock Solution for Substrate:** *(store up to 4 weeks at 4 ℃)*
47.5g Citric Acid
39g Na_2HPO_4
2.5g Urea peroxide
Add 1ℓ dH_2O
Adjust pH to 3.9

1.7.   **Using Solution for Substrate:** *(store up to 4 weeks at 4 ℃)*
100mℓ Stock Solution
Add 400mℓ dH_2O
Adjust pH to 3.8

1.8. **Tetramethylbenzedine Solution:** *(store up to 4 weeks in dark bottle at room temp)*
250mg 3,3'5,5'-Tetramethylbenzedine (TMB)
20mℓ Dimethylsulfoxide (DMSO)

1.9. **Substrate Solution:** *(use immediately & discard unused buffer)*
17mℓ Substrate using Solution
250µℓ TMB Solution

1.10. **Streptavidin Stock Solution:**
Add 2mℓ of Assay buffer to the 2mg Streptavidin (Sigma cat no:) to obtain a 1mg/ml solution
Distribute into 100µℓ aliquots in 1.5mℓ eppendorf tubes, seal with Parafilm, label and store at -20°C until further use.

1.11. **Streptavidin Using Solution:**
Add 900µℓ of Assay buffer to one of the 100µℓ Streptavidin aliquot made in step 1.10 to obtain a 0.1mg/ml solution (= 200µg/20µℓ)
Aliquot into 20µℓ aliquots in 0.5mℓ eppendorf tubes, seal with Parafilm, label and store at -20°C until further use.

1.12. **Streptavidin Solution:**
16mℓ Assay buffer
2000ng/20µℓ Streptavidin-POD-Conjugate.

1.13. **Stop reagent:**
2mol/ℓ H_2SO_4

2. **Antibody coating on microtitre plate (MTP):**
2.1. Label all plates to be coated on the short side with the date, Antibody type & personal ID.
2.2. Dispense 300µℓ antibody solution (1µg/well of Anti Rabbit IgG) to every well.
2.3. Stack plates no higher than 3 on top of each other and cover with cling film.
2.4. Incubate at 2-8 °C overnight (12-18 hours) in a fridge.
2.5. Discard the solution and refill each well with 300µℓ second coating buffer
2.6. Stack plates no higher than 3 on top of each other and cover with cling film.
2.7. Incubate at 2-8 °C overnight (12-18 hours) in a fridge.
2.8. Discard Second coating buffer, cover plates with cling film, and store at -20°C.

3. **Reagents (stock solutions – Standards, Second Antibody & Biotin label):**
Keep all stock solutions frozen at –20 °C until use. Freeze dried stock solutions can be kept at room temperature.
3.1. **Standards:**
See special instructions for each different Standard used.
3.2. **Second Antibody:**
See special instructions for each different antibody used.
3.3. **Biotin Label:**
See special instructions for each different biotin labelled steroid used.

4. **Assay Procedure:**
4.1. Before use wash coated MTP (made in step 2.) four times with washing solution (buffer 1.5.) in a wash bottle.
Remove the rest of liquid by pushing MTP on paper towels.

Label each plate on the long side with the Date, Assay type, Personal ID and Plate number.

4.2. Dispense 50μℓ of assay buffer for NSB and Zero STD in duplicate.
Dispense 50μℓ of each STD in duplicate.
Dispense 50μℓ of control in duplicate and
Dispense 50μℓ of each extracted faecal sample in duplicate.

4.3. Dispense 50μℓ of the biotin labelled steroid (diluted according to individual requirements – see step 3.3.) into each well.

4.4. Dispense 50μℓ antibody solution (diluted according to individual requirements – see step 3.2.) into each well **except use assay buffer for NSB, instead of antibody**.
Cover each MTP with Cling film incubate overnight at 4°C.

4.5. Decant incubated MTP and wash MTP four times with room temp washing solution.

4.6. Dispense 150μℓ of the Streptavidin enzyme solution (buffer 1.12.) in each well and incubate the plate covered in cling film & foil at room temp for 30 minutes by shaking.

4.8. Wash as in 4.5.

4.9. Dispense 150μℓ of the room temp substrate solution (buffer 1.9) in each well and incubate the plate covered in cling film & foil for 45 minutes at room temp by shaking.

4.9.1. Dispense 50μℓ of the stop reagent (buffer 1.13.) into each well. The blue colour turns yellow.

4.10. Read absorbance at 450 nm (reference filter, 620 nm).

APPENDIX C

LIST OF SAMPLES

IMMUNOREACTIVE PROGESTERONE CONCENTRATIONS IN
RHINO FAECES (NG PER G DRY WEIGHT)

Name of Individual	Date	Day	Prog conc. (ng/g DW)
Radimpe	21/10/2008	0	579.6
Radimpe	29/10/2008	8	522.6
Radimpe	10/11/2008	20	379.4
Radimpe	19/11/2008	29	368.2
Radimpe	26/11/2008	36	212.3
Radimpe	19/12/2008	59	79.0
Radimpe	22/12/2008	62	14.7
Radimpe	24/12/2008	64	21.8
Radimpe	04/01/2009	75	10.4
Radimpe	08/01/2009	79	7.8
Radimpe	12/01/2009	83	21.1
Motklaki	21/10/2008	0	543.3
Motklaki	06/11/2008	16	258.1
Motklaki	18/11/2008	28	251.5
Motklaki	25/11/2008	35	215.4
Motklaki	04/12/2008	44	208.2
Motklaki	16/12/2008	56	315.3
Motklaki	06/01/2009	77	203.9
Motklaki	08/01/2009	79	193.8
Motklaki	12/01/2009	83	280.0
Motklaki	16/01/2009	87	241.2
Motklaki	16/01/2009	87	295.8
Honkey	21/10/2008	0	7.1