NON-INVASIVE PROGESTAGENE MONITORING OF THE SOUTHERN WHITE RHINOCEROS *(CERATOTHERIUM SIMUM SIMUM)* AND A STUDY OF TIME DEPENDENT PROGESTAGENE DEGRADATION

DRS. M. M. MEIJERS
FACULTY OF VETERINARY SCIENCES, UTRECHT UNIVERSITY, THE NETHERLANDS, 2010

Correspondence to: Drs. M.M.Meijers, M.M.Meijers@students.uu.nl

Project engaged to the Institute for Breeding Rare and Endangered African Mammals (IBREAM), www.ibream.org

Supervised by: Dr. M. Paris, Dr. F. Dalerum & Dr. A. Ganswindt

Abstract

Although southern white rhinoceroses are the most numerous of the five rhinoceros species in the world, their population is declining and their status is near threatened. Since breeding is an important component in rhinoceros conservation, knowledge about the oestrus cycle should be available. The aim of this study was to get insight in the oestrus cycle of six free ranging southern white rhinoceroses in the Lapalala Wilderness, South Africa by measurement of faecal progestagene metabolites. This study, with duration of 3.5 months, is part of a long term study of IBREAM (Institute for Breeding Rare and Endangered African Mammals), Utrecht University and the University of Pretoria. Faecal samples were collected (from each animal 2-3 times a week) and progestagene measurements were conducted with an Enzyme Immunoassay (EIA). Luteal activity was detected in five out of six monitored rhinoceroses. Four rhinoceroses (Munyani, Mokibelo, Radimpe and Grikie) all showed different hormone profiles. Two animals showed a luteal phase: Munyani showed an oestrus cycle of around 68 days, Mokibelo also showed a luteal phase, but the length was undefined. The other two animals (Radimpe and Grikie) did not show any luteal phase in these 3.5 months. The profiles of the other two animals (Tharo and Mohklaki) were not analysed further due to low sample numbers.
A second aspect of this study was to visualize the degradation of progestagene metabolite concentrations in faecal samples over time. Samples for the degradation study were collected at 0, 0.15, 0.30, 1, 2, 4, 8, 16 and 32 hours after defecation from five different rhinoceroses. The study showed no significant effect of time on progestagene concentrations ($F(8,32) = 1.48$; $p = 0.202$). It can be concluded from this study that progestagene concentrations are not influenced by degradation in time from 0-32 hours after defecation.

1. Introduction

Nowadays there are five species of rhinoceros alive, the white rhinoceros (*ceratotherium simum ssp. cottoni and ssp. simum*), the black rhinoceros (d*iceros bicornis*), the Indian rhinoceros (*rhinoceros unicornis*), the Javan rhinoceros (*rhinoceros sondaicus*) and the Sumatran rhinoceros (d*icerorhinus sumatrensis)*. All five of the rhinoceros species are on the IUCN red list of threatened species. The status of the black rhinoceros, the Sumatran rhinoceros and the Javan rhinoceros is critically endangered. The Indian rhinoceros is vulnerable and the white rhinoceros status is near threatened (IUCN 2009. IUCN Red List of Threatened Species. Version 2009.1.). A taxon is Near Threatened when it has been evaluated against the criteria but does not qualify for Critically Endangered, Endangered or Vulnerable now, but is close to qualifying for or is likely to qualify for a threatened category in the near Future (IUCN Red List Categories and Criteria, Version 3.1, 2000).
The most serious threat for all different rhinoceros species populations is illegal hunting (poaching) for the international rhino horn trade. The demand for rhinoceros horn in the world is still high. The horn is being used as an ingredient in traditional medicine and as ornamental use in some countries (Owen-Smith, 1988).
Although the white rhinoceros is the most numerous of the five different species with a current population of 17.480 (IUCN) in the wild and 760 in captivity worldwide (IUCN), the commercial demand for rhinoceros horn and changes in political climate in African countries could lead to a serious decline in the white rhinoceros population, facing an uncertain future for them in the wild (Patton et al, 1999). Therefore the white rhinoceros population, although

only near threatened at present, is subject to attention and highly dependent on effective protection and intense conservation and management (Amin et al., 2003; Hermes et al., 2005). Successful breeding programmes are an important tool for rhinoceros conservation. The average gestation period of the white rhinoceros is around 16 months and adolescents become separated from their mothers when aged between 2 and 3.5 years. Modal birth intervals are 2.0-3.5 years with a mean of 2.6 years. It seems that white rhinoceros continue producing calves throughout their lifespan, but with advancing age, birth intervals lengthen while infant mortality increases (Owen Smith, 1988).
Limited information is available on the reproductive biology of white rhinoceroses and the data that do exist are conflicting, especially with regards to the oestrus cycle which implies more research is needed. Different cycle lengths were found, a shorter one (32.8 ± 1.2 days) and a longer one (70.1 ± 1.6 days) (Brown et al., 2001). Patton et al. (1999) also found two different cycle types, type 1 approximately 1 month in duration and type 2 approximately 2 months in duration. The two cycle types had similar interluteal phase lengths, but type 2 cycles were characterized by extended luteal phases lasting more than twice as long as type 1 luteal phases. The type 1 cycles were characterized as the typical reproductive cycle for the white rhinoceros and the long cycles were designated abnormal because of specific pathological factors (Radcliff et al., 1997).
Like Brown et al. (2001), most studies regarding the oestrus cycle were conducted on captive white rhinoceroses. Little or conflicting data can be found about the oestrus cycle in free ranging white rhinoceroses and its attendant circumstances, which are important to take into consideration (like sample collection and identifying individual rhinoceroses). A study on free ranging rhinoceroses needs to be non-invasive and as the former researchers on this project (A.C. van der Goot, Y.N. Charbon and B. Bitter) described progestagene measurements of faecal samples give good results regarding reproductive activity. Only little is known about the degradation processes of progestagens in faeces. A study of Neumann et al (2002) describes the stability of progestagens in faeces of different free ranging animals, including rhinoceroses, in relation to several external factors such as the dry mass of faeces, time point of freezing, duration of storage of the frozen samples and multiple defrosting of the samples. Progestagene concentration in relation to time point of storage (directly frozen or frozen after storage for 24 or 48 hours in room temperature) showed a significant increase, when not stored directly, in rhinoceroses. Hormone degradation is thus an important aspect to consider in research on the oestrus cycle of free ranging animals, as it will help to decide what samples are reliable to include in the overall analysis (a fresh sample compared to an older sample).
Against this background, this study will have two aspects. The first aim of this study is to analyze the oestrus cycle length of free ranging white rhinoceroses over a long time (2 years) period. This current report will describe a 3.5 month period of the long time research on the oestrus cycle.
The second aim of this study is to study the effect of time on the degradation of progestagene levels in faeces during a 32 hour period following defecation, the hypothesis of this study is to find a difference in the progestagene concentration over time, either an increase or a decrease.

2. Materials and methods

2.1 Study site and animals

The area where this study was performed is called the Lapalala Wilderness, one of the largest private game reserves in the Limpopo province of South Africa. Lapalala falls within the summer rainfall region with a mid-summer (January) seasonality. The overall mean annual rainfall for Lapalala is estimated at 500 mm. Mean annual rainfall is lower in the low-lying area in the north (400 mm) than in the higher lying south-western border (600 mm). The

temperatures reach peak values during summertime, January (mean maximum temperature is 30°C) and lowest values during wintertime, July (mean maximum temperature is 20°C), in which frost is common (Lapalala Wilderness. Lapalala.com).
The southern white rhinoceros has successfully been introduced in the Lapalala Wilderness. This 36.000 ha enclosed reserve provides a sanctuary for the breeding of endangered animals, and forms a good area for basic reproductive performance investigation in rhinoceroses. The individual rhinoceroses can be located for non-invasive faecal sampling by a ranger who is familiar with this area and has specific knowledge of the home range of each individual rhinoceros. Since 2001 the reserve is no longer open to tourists and the animals live freely with minimal human interaction. At present there are approximately 40 white rhinoceros living in this reserve.
The animals investigated in this study were six female southern white rhinoceroses that were accurately identified for this study by the previous researchers of this long time project. Five of the animals were proven breeders and all had a calf with them during the study period. One of the included animals was a 5 year old calf of one of the other study animals, that supposedly was reproductively active based on her age (white rhinoceroses start to show follicular activity at an age of three to four years; Hermes et al. 2006, Owen Smith, 1988).
Two of the study rhinoceroses (Mohklaki and Tharo) were hard to find and samples could not been taken regularly, they were excluded from the project halfway the study to secure the sample collection frequency of the other animals.

Table 1 - Summary of animals used in this study.

Study animal	Age (yrs)	Age of youngest calf (yrs)	Number of calves
Munyani	13.5	5.7	2
Mokibelo	5.7	/	/
Grikie	20.6	2	5
Radimpe	14.3	<1	3
Mohklaki	15.7	<1	4
Tharo	16.6	<1	3

2.2. Sample collection
The aim of sample collection was to get 2-3 samples of all the rhinoceroses included in the project every week to get a good view on the oestrus cycle. Because two of the animals (Mohklaki and Tharo) were not find as frequent as necessary, the two rhinoceroses were excluded from the study due to low sample size (respectively: n = 8 and n = 7).
To collect the samples rhinoceroses had to be found, identified and subsequently they had to defecate so sample collection could take place. To find the animal there was an experienced guide who knew the area, the individual animal and the specific detailed home range of every rhinoceros in the study. As white rhinoceroses show bimodal activity patterns with main active periods early in the morning and late in the afternoon (Owen Smith, 1988), searching for them has been done twice a day during those times. The search started with driving through the home range of the animal of interest, searching for fresh footprints and from that point tracking the rhinoceros on foot. Identification was carried out at three levels; the mother, the mother-calf combination and the company of other rhinoceros. The characteristics which were used at all levels were footprints, ear-notches and other physical characteristics of the animal of interest.
Samples were taken either fresh or on the track. Samples were called fresh when animal seen defecating. Fresh samples were preferable but if samples were taken on the track, identifying the rhinoceros took place after collecting the sample by following her track. Collection was carried out with gloves (Hartmann Peha-soft, REF:942150) from the inside of the faecal bolus. 10-40 gram faeces was collected into a glass container, avoiding contamination and

removing most of the indigested material. The difference in reliability of the collected samples was documented (either collected fresh or on the track). Furthermore documentation on group members seen together with the study animals was made. This could give information on group behaviour and may therefore aid in finding rhinoceroses in the future.
After collecting the samples they were stored in a cooler until arriving back at the base, where they were stored in the permanent freezer at -18 ˚C.
For the degradation study five animals were selected. Samples for the degradation experiment were collected at the same way as described for the long term study but only fresh samples were included in this study. Faecal samples were collected at different time points: 0, 0.15, 0.30, 1, 2, 4, 8, 16 and 32 hours after defecation. After the first sample at t = 0, 2-3 pellets were brought to the base and stored outside in the shade, where the remainder of the samples was taken in the same way.

a. b. c.

Photograph 1- a. Footprint of a rhinoceros used for tracing them, b. Munyani and Mokibelo identified, c. Sample collection.

2.3. Fecal extraction

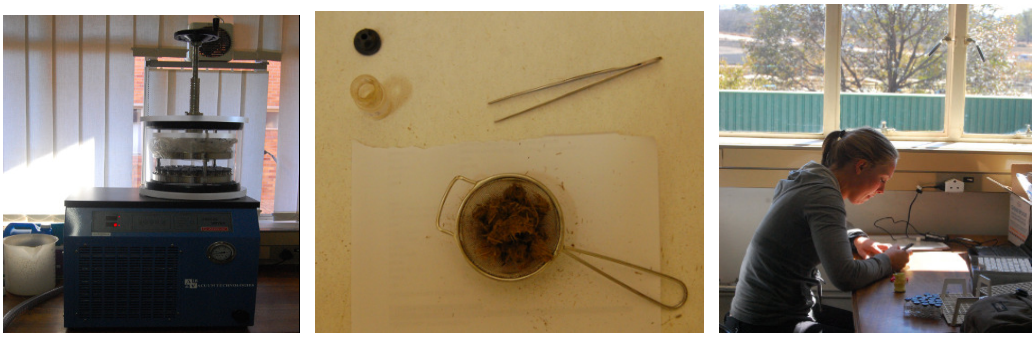
Extraction of the progesterone metabolites from the faecal samples was done conform the 'Standard Operating Procedure – Extraction Method for Dry Faecal Samples' of the Onderstepoort Veterinary Institute in Pretoria (appendix A). The samples were freeze dried for 48 hours in a vacuum oven (Instruvac Freeze-drier from Air & Vacuum Technologies, Pretoria, South Africa, Model: VFDT 02.50) at – 50 ºC and 80-90 mTorr in order to get the liquid out of the samples to prevent variability in weight. After freeze drying pulverization of the samples took place using a small sieve and a set of tweezers to separate the small powder from the fibers. To prevent cross contamination the surface and the utensils were cleaned using 80% EtOH (prepared from Ethanol Absolute 99%, Merck, Saarchem, diluted with distilled water) between each sample. Approximately 50 mg (in between 50 and 55 mg) was weighed out of every sample and poured into a sample tube. The precise weight of every sample was noted and used to calculate the exact progestagen levels per gram dry weight. Three milliliter of 80% EtOH was added to each sample tube and all the tubes were vortexed on high speed for 15 minutes. Thereafter the tubes were centrifuged on 3300 rpm for 10 minutes. The supernatant was transferred into an eppendorf tube and stored at -20˚C, ready for the Enzyme Immunoassay (EIA).

2.4. Enzyme immunoassay

Samples were analysed using a microtitreplate enzyme immunoassay. Progestagene concentration was quantified by an enzyme immunoassay (EIA) (Szdzuy, et al, 2006) using a polyclonal antibody (rabbit) against 5α-pregnan-3β-ol-20-one-3-HS-BSA and 5α-pregnan-3β-ol-20-one-3-HS-peroxidase label. The cross-reactivities to other progesterone metabolites tested by the 5α-pregnan-3β-ol-20-one EIA were as follows: 5α-pregnan-3-α-ol-20-one, 650%; 5α-pregnan-3β-ol-20-one, 100%; 4-pregnen-3,20-dione (progestagene) 72%; 5α-pregnan-3,20-dione, 22%; <0,1% for 5β-pregnan-3α,20α-diol, 4-pregnen-20α-ol-3-one, 5β-

pregnan-3α-ol-20-one, 5α-pregnan-20α-ol-3-one, 5α-pregnan-3β,20α-diol and 5α-pregnan-3α,20α-diol.

a. b. c.



Photograph 2- a. Samples in Instruvac Freeze-drier, b. Pulverization of freeze-dried material, c. Sample registration.

2.5. Data analysis

Progestagene concentrations are given in µg/g dry weight and are presented as mean ± standard deviation (SD) for each animal. Progestagene metabolite concentrations are described (in the results section) from the 1st of May until the 15th of August.

Classification of the rhinoceroses in this study into four groups was carried out in the same way as described by Schwarzenberger et al. (1998), but only based on duration and regularity of the oestrus cycle. Schwarzenberger et al (1998) classified rhinoceroses into four major categories based on the oestrus cycle length and the luteal phase concentrations: 1. regular oestrous cycles of 10 weeks duration; 2. oestrous cycles between 4–10 weeks; 3. no apparent cycle regularity, but luteal activity indicated; 4. no apparent luteal activity.

The follicular and luteal phase of the animals were determined following the method described by Brown et al 2001; a non pregnant baseline was calculated using an iterative process in which values that exceeded the mean plus 1.5 SD were excluded. The average was then recalculated and the elimination process was repeated until no values exceeded the mean plus 1.5 SD. The start of the luteal phase was defined as the first value that increased above the baseline by 50 % and thereafter remained elevated for at least 2 consecutive weeks. The end of the luteal phase was defined as the first of two consecutive values that returned to baseline concentrations.

To check if progestagene concentrations changed over time, degradation samples were analysed with a General Linear Model (GLM), repeated measures Analysis of Variance (ANOVA), with time as a repeated measure.

2.6. Longitudinal study

This study is a component of a long term study on the oestrus cycle of free ranging white rhinoceroses in the Lapalala Wilderness and is conducted from May 2009 until August 2009. Other researchers in this long term study are Ms Y.N. Charbon, Ms A.C.van der Goot, Ms B. Bitter and Ms J.S. Swinkels and they collected samples from October 2008 till December 2009.

3. Results

3.1. Longitudinal study

Table 2 shows the means ± SD of progestagene concentrations of the study animals. As shown in this table the number of samples from Mohklaki and Tharo are too small to make a

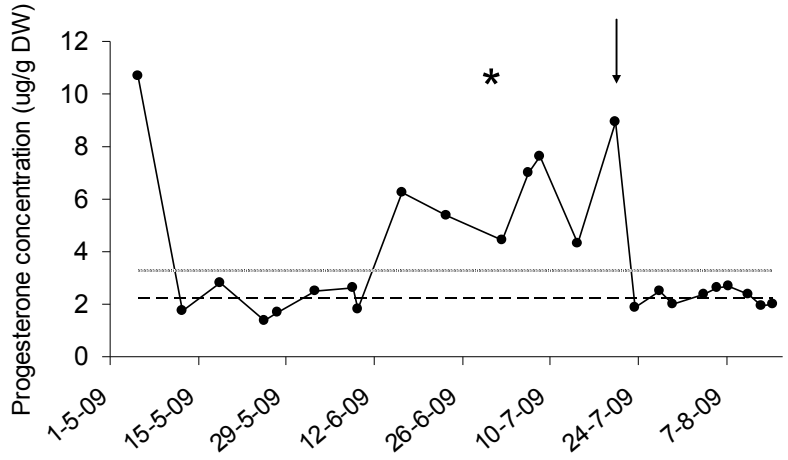
reliable proclamation about their oestrus cycle. Following the classification from Schwarzenberger based on oestrus cycle duration these two rhinoceroses should be classified as type 3 or 4 animals (no oestrus cycle detected).
From the moment these two animals were excluded from the study sample frequency increased in the other rhinoceroses, especially in Munyani, Mokibelo and Radimpe, as shown in figure 1.

Table 2 - Descriptive data of progestagene concentrations (mean ± SD in µg/g dry weight and baseline values) for each animal separately during the sampling period

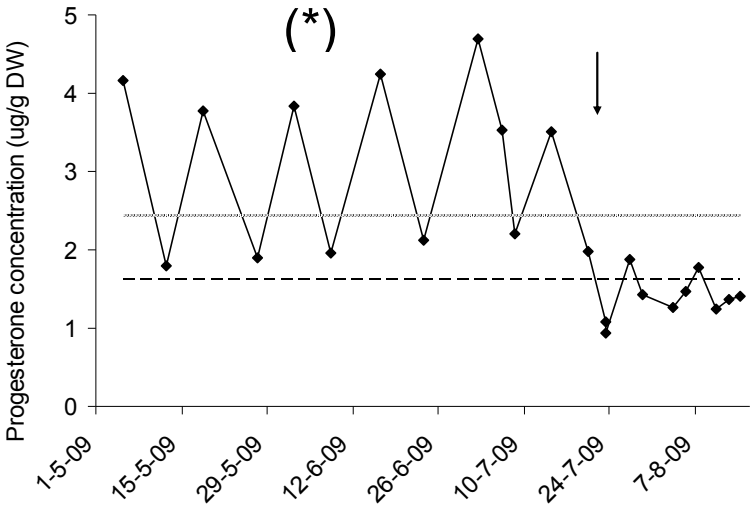
Rhinoceros	No samples	Mean (µg/g)	SD (µg/g)	Baseline	50% ↑ Baseline
Munyani	24	3.73	2.57	2.18	3.28
Mokibelo	23	2.33	1.17	1.61	2.42
Radimpe	16	2.07	0.43	1.83	2.74
Grikie	18	0.69	0.18	0.59	0.89
Mohklaki	8	2.4	0.8	2.03	3.04
Tharo	7	3.48	0.8	3.48	5.22

.
According to classification system following Schwarzenberger, based on oestrus cycle duration, the other four rhinoceroses were categorized as follows: Munyani and Mokibelo; type 1 or 2 animals and Radimpe and Grikie; type 3 or 4 rhinoceroses. However, differences in mean progestagene levels are seen amongst the various rhinoceroses, especially in Grikie who has a low mean concentration.
Both rhinoceroses Grikie (fig 1d) and Radimpe (fig 1c) do show single measurements of progestagene elevations of 50 % above the baseline but no elevation for a consecutive time period. This indicates that there was luteal activity. But no luteal phase in this period of sampling. Mokibelo (fig 1b) shows a different pattern in her progestagene concentrations, the first part shows several fluctuations with a mean value above 50 % of the baseline. This probably indicates a luteal phase with random fluctuations. The last part of the sampling period shows concentrations around the baseline, indicating a part of the follicular phase. Munyani (fig 1a) demonstrates a clearer pattern in her progestagene concentrations, where follicular and luteal phases can be distinguished. An oestrus cycle can be recognized, with concentrations around the baseline for 31 days and levels of 50% above the baseline for 37 days. This indicates an oestrus cycle of around 68 days.

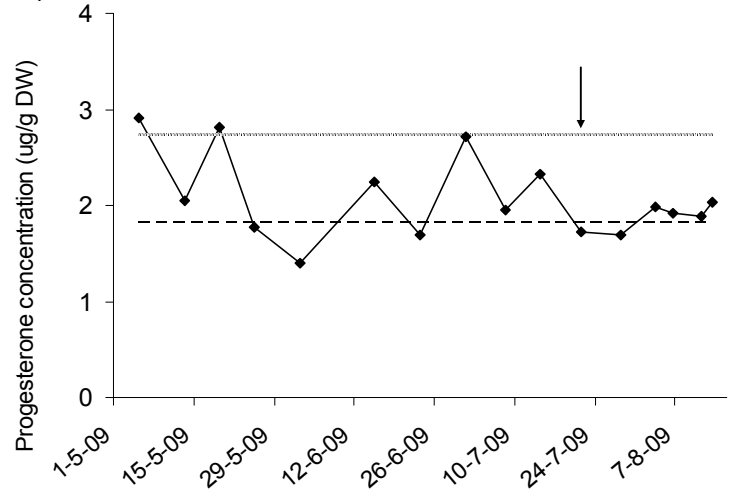
a)



b)


(*)
Progesterone concentration (ug/g DW)
0
1
2
3
4
5
1-5-09
15-5-09
29-5-09
12-6-09
26-6-09
10-7-09
24-7-09
7-8-09

c)


Progesterone concentration (ug/g DW)
0
1
2
3
4
1-5-09
15-5-09
29-5-09
12-6-09
26-6-09
10-7-09
24-7-09
7-8-09

d)

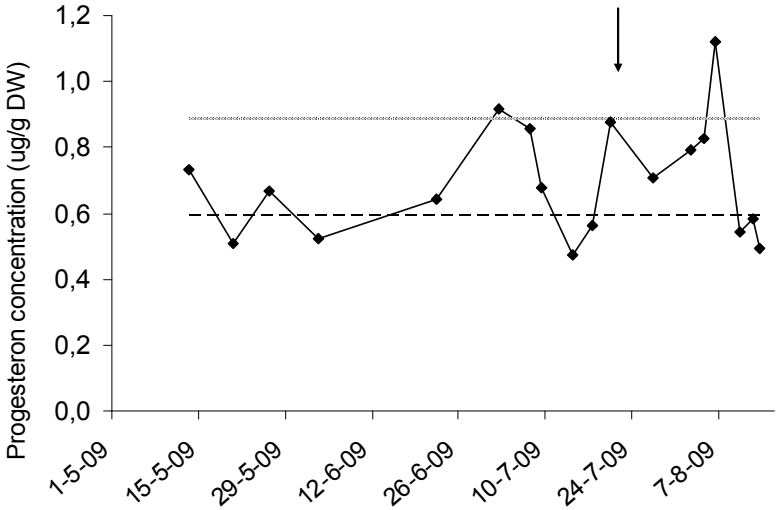

Progesteron concentration (ug/g DW)
0,0
0,2
0,4
0,6
0,8
1,0
1,2
1-5-09
15-5-09
29-5-09
12-6-09
26-6-09
10-7-09
24-7-09
7-8-09

Figure 1 - Individual profiles of faecal progestagene concentrations of respectively Munyani (a), Mokibelo (b), Radimpe (c) and Grikie (d) during sampling period from May until August, arrow indicates increase in sample frequency after excluding two rhinoceroses, striped line is progestagene baseline, dotted line is an increase of 50% of the baseline and * indicates the luteal phase.

3.2. Degradation study

There was no significant effect of time on progestagene concentration ($F(8,32) = 1.48$; $p = 0.202$). All five rhinoceroses included in this study showed minimal fluctuations in the progestagene concentration over time. Three rhinoceroses showed concentrations of around 2 µg/g dry weight, one showed a lower concentration (< 1 µg/g dry weight) and one of them showed a progestagene concentration of around 6 µg/g dry weight. Figure 3 shows the degradation profiles of progestagene concentrations of the five rhinoceroses included in this study.

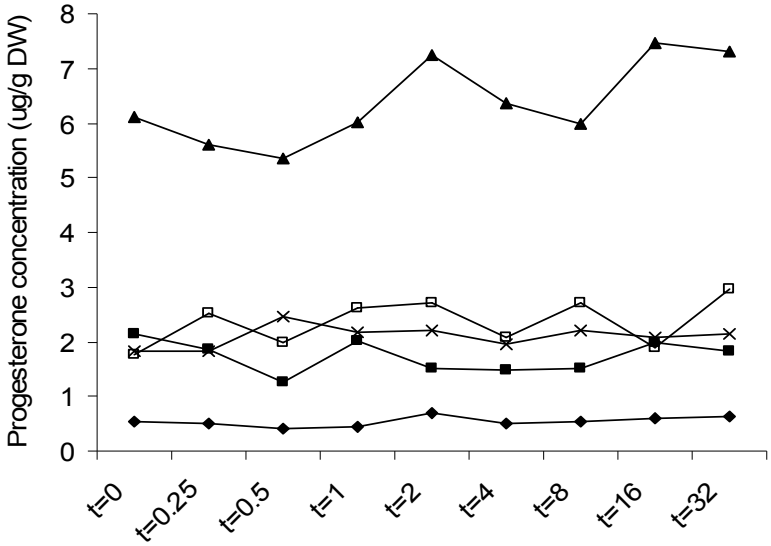


Figure 3 - Progestagene degradation of five rhinoceroses.

4. Discussion

4.1. Longitudinal study

A longitudinal study on free ranging female rhinoceroses has generally some limitations. First of all the aim of getting 2-3 samples from each animal every week is difficult just because of finding all of the animals every time of the year, because vegetation and therefore visibility changes dramatically over the year. By reducing the number of study animals from six to four animals sample frequency increased, because of less time in between finding them a more specific search could be carried out in the right direction. But on the other hand a reduction in animal number reduces the quality of the desired data set as well. During seasons rhinoceroses behave differently because of grass offer, in dry periods animals get additional food and concentrate themselves in larger groups around the feeding spot, at which time finding individual animals is easier. Meanwhile during the 3.5 months of this study period the rhinoceroses did not get additional food which made it harder to trace them on time. Sometimes animals were found late in the afternoon and because of the dusk waiting for defecation had to be ended and no samples could be collected. A system as described by A.C.

van der Goot (2009), VHF telemetry can be of great value in finding the rhinoceroses and by implementing this, the sample frequency rate could be increased in the future.
The results of this study indicates that faecal progestagene measuring in free ranging white rhinoceroses is a good method to determine the oestrus status, only a longer study period is needed to get more insight into the hormone profiles. The study provides insight into the progestagene concentrations during the research period (May until August) of six rhinoceroses. Five out of six animals showed luteal activity. However two animals were excluded from further analysis due to low sample numbers. The other four rhinoceroses all showed different patterns.
The progestagene profile of Grikie shows two points which are elevated above the 50 % baseline. This can be due to lack of reliability of the samples because both samples were collected on the track instead of fresh, which means there is a small change that it was not her sample. According to her profile those points indicate luteal activity. Nevertheless no luteal phase can be determined in this profile, indicating that this animal was in anoestrus or was in the follicular phase of the oestrus cycle during this study. The first explanation is most likely, because she shows low values of progestagene (baseline 0.59) and in other studies, like Brown et al 2001, low values indicate an anoestrus period and also a follicular phase of 3.5 months seems not likely.
Like Grikie, Radimpe also does not show a consecutive elevation of progestagene concentrations above the 50 % baseline. She shows two points above the 50% baseline, which indicate luteal activity. Reliability of the samples is for the first elevated point high and for the second point like Grikie's elevated samples. Radimpe's baseline is placed higher compared to Grikie's during this period (1.83) which possibly means she is cyclic (follicular phase), but because no luteal phase took place during the sampling period and a follicular phase of 3.5 months is not likely an anoestrus period during this study is for Radimpe also more adequate.
The progestagene profile of Mokibelo shows numerous fluctuations in the first part of the study, the fluctuations are not due to different times on the day of sample collection or due to other differences in sample collection or handling. Therefore the fluctuations are probably random or caused by another unknown aspect. When ignoring fluctuations and calculating the mean concentration of this period, which is 3.06, this value reaches above the 50 % baseline. This can give an indication for a luteal phase in this period. Subsequent to the fluctuating points, concentrations are found around the baseline which can possibly indicate a follicular phase. No conclusion can be drawn about the length of both phases because of the timing of this study (luteal phase already started before sampling took place and follicular phase did not end before sampling stopped).
Munyani showed a clearer progestagene profile with one oestrus cycle in the sampling period. From the start of the study concentrations appear from a high progestagene level onto the baseline for 31 days from where they elevate above the 50 % baseline for 37 days until they go back to baseline levels until the end of the sampling period. This process indicates an oestrus cycle from around 68 days which compared to literature (Brown et al, 2001) can be described as a 'long' cycle. According to Brown et al a long cycle is not a typical reproductive cycle for the white rhinoceros, but opinions are not clear and no consensus exists at this point.
To get more knowledge about the oestrus cycle in free ranging white rhinoceroses it would be advisable to have data available for longer than 3.5 months. These data should be comparable mutually so the rhinoceroses can be followed in time and more reliable statements can be made about the oestrus cycle.
Classifying rhinoceroses in different categories gives a good impression about their hormonal status, but to classify animals into different categories on oestrus cycle duration and progestagene levels as described by Schwarzenberger et al. (1998), the methods used for

progestagene quantification should be exactly the same. This study used another EIA, and therefore the absolute progestagene values are not comparable. This means that the classification system of Schwarzenberger cannot be unequivocally used to classify the rhinoceroses and there cycling pattern based on the progestagene levels revealed in this study.

4.2. Degradation study

Most studies regarding the oestrus cycle of southern white rhinoceroses are conducted on captive animals (Brown et al., 2001, Schwarzenberger et al., 1998), which makes sample collection easier compared to free ranging animals. In captivity no search for the animal is needed and it is easier to get a fresh sample. The study described here include free ranging white rhinoceroses, samples were sometimes found on unknown time after defecation, and to maximize sample frequency it would be desirable to be able to include these non-fresh samples in the study as well. The degradation study showed that within 32 hours after defecation progestagene concentrations in faeces do not significantly differ. This means that older samples (< 32 hour after defecation) are as reliable as fresh samples regarding the progestagene concentration. Regarding the right sample from the right animal it is important to identify the rhinoceros accurately after sampling takes place.

Rhinoceroses included in this study had their home range at different distances from the base; therefore time from collecting the sample to freezing was different from each animal and therefore time (sampling to freezing) in between each sample from each individual animal corresponded.

Samples were stored at the base in the shade on outside temperatures and not all samples were taken on the same day, so not all variables were equal. Despite the differences in these values progestagene concentrations in all animals were not significantly different during time. This indicates that variable factors such as temperature do not affect progestagene concentrations in faeces during time. Also shade or sunshine will probably not have an influence on progestagene concentrations, because a sample is taken from the inside of the faecal pellet. To test this hypothesis further research is needed.

5. Conclusion

5.1. Longitudinal study

- To get sufficient knowledge about the oestrus cycle in free ranging southern white rhinoceroses this study indicates that studying faecal progestagene levels is a reliable method.
- Describing progestagene concentrations following a non pregnant baseline gives a good insight into the cycling pattern.
- A classification system for this specific type of EIA based on both concentrations and oestrus cycle duration would be an advantage and should be therefore developed.
- To be able to compare hormone data revealed in different parts of the study, the samples test system should be used for the analysis of all samples.

5.2. Degradation study

- The current degradation study indicates that progestagene concentrations in collected faecal samples are not influenced by time before collection, a non-fresh (<32 hour after defecation) samples can be collected without a problem in reliability of progestagene concentrations and included in future studies.

6. Acknowledgements

I would like to thank a lot of people who made this research possible and who helped me in performing this study. First of all I would like to thank the Institute for Breeding Rare and Endangered African Mammals (IBREAM), and my supervisors Dr. Monique Paris, Dr. Frederik Dalerum and Dr. André Ganswindt who made this research possible and assisted me by transforming ideas into actual actions. I would also like to thank the staff members of the Veterinary Hormone Lab, Dept: PAS, Section: Reproduction/Veterinary Wildlife Studies, Faculty of Veterinary Science, University of Pretoria: Stefanie Muenscher and Dr. André Ganswindt, for helping me by analyzing the collected data. A great thanks goes to the management of the Lapalala Wilderness: Duncan Parker, Mike Gregor, Anton Walker, Roger Collinson, Anthony and Erin Roberts and all the other terrific staff and rangers for the stay and help in the fantastic Lapalala Wilderness. My special thanks goes to Thomas Litshani who is the best ranger and helped us everyday eager in our search for the rhinoceroses. Last but not least a want to thank my co-worker Chrispijn Schilp for supporting me on this study when possible and Sandra Cornelisse who assisted me with the statistic analysis.

7. References

Amin, R., Bramer, M. and Emslie, R. 2003. Intelligent data analysis for conservation: experiments with rhino horn fingerprint identification, Knowledge-Based Systems, volume 16, issue 5-6, pp. 329-336.

Amin, R., Emslie, T. K., Foose, R.H., and van Strien, N. 2006. An overview of the conservation status of and threats to rhinoceros species in the wild. International Zoo Yearbook, volume 40, number 1, pp. 96-117.

Berkeley, E.V., Kirkpatrick, J.F., Schaffer, N.E., Bryant, W.M. and Threlfall, W.R. 1997. Serum and fecal steroid analysis of ovulation, pregnancy and parturition in the black rhinoceros (*Diceros bicornis*). Zoo Biology, number 16, pp. 121–132.

Bertschinger, H.J. 1994. Reproduction in black and white rhinos: a review. Proceedings of a symposium on "Rhinos as Game Ranch Animals", pp. 96-99.

Bitter, B. 2009. Non-invasive reproductive monitoring of six wild female white rhinoceroses (*Ceratotherium simum simum*).

Brown , J. L., Bellem, A.C., Fouraker , M., Wildt , and D.E., Roth, T. L. 2001. Comparative analysis of gonadal and adrenal activity in the black and white rhinoceros in North America by noninvasive endocrine monitoring. Zoo Biology, volume 20, issue 6, pp. 463 – 486.

Charbon Y.N. 2009. Determining the reproductive status of 3 female free-ranging white rhinoceros (Ceratotherium simum simum) by measuring faecal progestagen levels.

Goot, van der, A. 2009. An analysis on cyclicity and pregnancy in the Southern white rhinoceros *(Ceratotherium simum simum)* by noninvasive progesterone monitoring using VHF radio telemetry.

Graham, L., Schwarzenberger, F., Möstl, E., Galama, and W., Savage, A. 2001. A versatile enzyme immunoassay for the determination of progestogens in feces and serum. Zoo Biology, volume 20, issue 3, pp. 227 – 236.

Hermes, R., Hildebrandt, T. B., Walzer, C., Göritz, F., Patton, M. L., Silinski, S., Anderson, M. J., Reid, C.E., Wibbelt, G., Tomasova, K., and Schwarzenberger, F. 2006. The effect of long non-reproductive periods on the genital health in captive female white rhinoceroses. (*Ceratotherium simum simum*, *C.s. cottoni*). Theriogenology, volume 65, issue 8, pp. 1492-1515.

Hermes, R., Hildebrandt, T. B., Blottner, S., Walzer, C., Silinski, S., Patton, M.L., Wibbelt, G., Schwarzenberger, F. and Göritz, F. (2005). Reproductive soundness of captive southern and northern white rhinoceroses (Ceratotherium simum simum, C.s. cottoni): evaluation of male genital tract morphology and semen quality before and after cryopreservation, Theriogenology, volume 63, issue 1, pp. 219-238.

Hindle, J.E. and Hodges, J.K. 1990. Metabolism of oestradiol-17β and progestagen in the white rhinoceros (*Ceratotherium simum simum*). Journal of Reproduction and Fertility, issue 90, pp. 571–580.

IUCN 2009. IUCN Red List of Threatened Species. Version 2009.1, Available at: www.iucnredlist.org.

IUCN 2009. Red List Categories and Criteria, Version 3.1, 2000.

Neuman, G., Gottschalk, J., Eulenberger, K., Grün, F. 2002. The stability of progestagene in feces of different wild animals kept in a zoological garden. Deutsch Tierartztl Wochenschrift, issue 109(5), pp. 245-249.

Lapalala Wilderness. Available at: www.lapalala.com.

Owen-Smith, R.N. 1988. Megaherbivores: The Influence of Very Large Body Size on Ecology. Cambridge Univ. Press, Cambridge, UK.

Patton, M.L., Swaisgood, R.R., Czekala, N.M., White, A.M., Fetter, G.A., Montagne, J.P. and Rieches, R.G. 1999. Reproductive cycle length and pregnancy in the southern white rhinoceroses (*Ceratotherium simum simum*) as determined by fecal pregnane analysis and observations of mating behavior. Zoo Biology, issue 18, pp. 111–127.

Radcliffe, R.W., Czekala, N.M. and Osofsky, S.A. 1997. Combined serial ultrasonography and fecal progestin analysis for reproductive evaluation of the female white rhinoceros (*Ceratotherium simum simum*): preliminary results. Zoo Biology, issue 16, pp. 445–456.

Roth, T.L. 2006. A review of the reproductive physiology of rhinoceros species in captivity. International Zoo Yearbook, volume 40, number 1, pp. 130-143.

Schwarzenberger, F., Walzer, C., Tomasova, K., Vahala, J., Meister, J. and Goodrowe, K.L. 1998. Faecal progestagen metabolite analysis for non-invasive monitoring of reproductive function in the white rhinoceroses (*Ceratotherium simum*). Animal Reproduction and Science, issue 53, pp. 173–190.

Szdzuy, K., Denhard, M., Strauss, G., Eulenberger, K. and Hofer, H. 2006. Behavioural and endocrinological parameters of female African and Asian elephants (*Loxodonta africana* and *Elephas maximus).* International Zoo Yearbook *,* volume 40, number 1, pp. 41–50.

Appendix A

STANDARD OPERATING PROCEDURE

Extraction Method for Dry Faecal Samples

University of Pretoria, South Africa

Phase 1 – Pulverize

1. **Freeze-dried sample vials, sealed and kept in freezer (remove in batches of 10)**
2. **70 - 80 % Ethanol.**
3. **Gloves, mask, lab coat, tissues, waste paper, paper towel roll, scissors, sieve, tweezers, dustbin bag, list of samples.**

NB: All phases -Avoid cross-contamination! Change gloves, regularly, clean work surface and utensils in between EACH sample!

1. Clean work surface, and all utensils with EtOH.
2. Break seal of of vial; remove faecal matter carefully using tweezers. Place into sieve that is positioned over waste paper.
3. Scratch matter around; allow only fine powder to fall through.
4. Discard coarse matter onto tissue paper.
5. Fold paper with powdered matter into a funnel, and place sample back into the vial.
6. Close tightly and tick the sample number on the list.
7. Soak tweezers in EtOH, wipe sieve clean.
8. Store at room temperature in marked boxes until all samples are done.

Phase 2 – Weigh

1. **Labels, permanent marker, gloves, tissue paper, small spatula, 70-80% EtOH.**
2. **Sample tubes with caps and list of samples. Polystyrene rack.**
3. **Scale (3 decimal).**
4. **Waste paper.**

1. Remove sample vials in bathes of 10. Label sample tubes.
2. Remove cap from sample tube and place tube onto scale. Zero the reading.
3. Wipe soaked spatula clean, remove powdered feacal matter carefully and place into sample tube. Weigh **0.05g** sample (not more than 0.055g and not less than 0.05g).
4. Record actual weight on list and tick sample off on list.
5. Cap tube and place in polystyrene rack.
6. Clean balance after use.

Phase 3 – Final Separation

1. **80 % EtOH in Schott bottle, 5ml pipette and tips.**
2. **Centrifuge tube with cap (1.5 – 2ml)**
3. **Centrifuge, Multi-shaker, plastic test tube rack.**
4. **Polystyrene rack and list of samples.**

1. Prepare enough 80% Ethanol for 3ml per sample (must be freshly prepared). Work in bathes of 72 samples at a time. Place in test tube rack.
2. Add 3ml EtOH to each tube, close immediately. *AVOID touching inside of tubes!*
3. Place full rack with 72 tubes onto multi-shaker on high speed for 15 minutes.
4. Label a centrifuge tube and tick sample off on list.
5. Centrifuge at 3000 rpm for 10 minutes.
6. Remove 1.5 to 2ml **clear** supernatant from tube with pipette, transfer to centrifuge tube.
7. Store **upright** at -20°C. Sample ready for ELISA.

Appendix B

LIST OF SAMPLES

IMMUNOREACTIVE PROGESTAGENE CONCENTRATIONS IN
RHINO FAECES (µG PER G DRY WEIGHT)

Longitudinal study

Rhino	Date	Prog.conc. (ug/g DW)
Grikie	2009/05/13	**0.73**
Grikie	2009/05/20	**0.51**
Grikie	2009/05/26	**0.67**
Grikie	2009/06/03	**0.52**
Grikie	2009/06/22	**0.64**
Grikie	2009/07/02	**0.92**
Grikie	2009/07/07	**0.86**
Grikie	2009/07/09	**0.68**
Grikie	2009/07/14	**0.47**
Grikie	2009/07/17	**0.56**
Grikie	2009/07/20	**0.88**
Grikie	2009/07/27	**0.71**
Grikie	2009/08/02	**0.79**
Grikie	2009/08/04	**0.83**
Grikie	2009/08/06	**1.12**
Grikie	2009/08/10	**0.54**
Grikie	2009/08/12	**0.58**
Grikie	2009/08/13	**0.50**
Mohlaki	2009/05/22	**3.85**
Mohlaki	2009/05/30	**2.34**
Mohlaki	2009/06/19	**1.69**
Mohlaki	2009/06/25	**2.38**
Mohlaki	2009/07/03	**3.22**
Mohlaki	2009/07/08	**2.40**
Mohlaki	2009/07/09	**1.38**
Mohlaki	2009/07/13	**1.97**
Mokibelo	2009/05/05	**4.16**
Mokibelo	2009/05/12	**1.79**
Mokibelo	2009/05/18	**3.77**
Mokibelo	2009/05/27	**1.90**
Mokibelo	2009/06/02	**3.84**
Mokibelo	2009/06/08	**1.97**
Mokibelo	2009/06/16	**4.25**
Mokibelo	2009/06/23	**2.13**
Mokibelo	2009/07/02	**4.70**
Mokibelo	2009/07/06	**3.53**
Mokibelo	2009/07/08	**2.19**
Mokibelo	2009/07/14	**3.51**
Mokibelo	2009/07/20	**1.98**
Mokibelo	2009/07/23	**1.08**

Degradation study

Rhino	Date	Prog.conc. (ug/g DW)
Grikie	2009/08/10	**0.54**
Grikie	2009/08/10	**0.51**
Grikie	2009/08/10	**0.42**
Grikie	2009/08/10	**0.45**
Grikie	2009/08/10	**0.68**
Grikie	2009/08/10	**0.49**
Grikie	2009/08/10	**0.54**
Grikie	2009/08/10	**0.60**
Grikie	2009/08/10	**0.64**
Mokibelo	2009/06/23	**2.13**
Mokibelo	2009/06/23	**1.87**
Mokibelo	2009/06/23	**1.25**
Mokibelo	2009/06/23	**2.00**
Mokibelo	2009/06/23	**1.52**
Mokibelo	2009/06/23	**1.49**
Mokibelo	2009/06/23	**1.51**
Mokibelo	2009/06/23	**1.99**
Mokibelo	2009/06/23	**1.82**
Munyani	2009/06/23	**6.11**
Munyani	2009/06/23	**5.61**
Munyani	2009/06/23	**5.34**
Munyani	2009/06/23	**6.00**
Munyani	2009/06/23	**7.24**
Munyani	2009/06/23	**6.37**
Munyani	2009/06/23	**5.98**
Munyani	2009/06/23	**7.47**
Munyani	2009/06/23	**7.32**
Radimpe	2009/07/28	**1.83**
Radimpe	2009/07/28	**1.83**
Radimpe	2009/07/28	**2.45**
Radimpe	2009/07/28	**2.17**
Radimpe	2009/07/28	**2.19**
Radimpe	2009/07/28	**1.97**
Radimpe	2009/07/28	**2.22**
Radimpe	2009/07/28	**2.07**
Radimpe	2009/07/28	**2.14**
Pedi	2009/08/13	**1.76**
Pedi	2009/08/13	**2.53**
Pedi	2009/08/13	**1.97**
Pedi	2009/08/13	**2.61**

Mokibelo	2009/07/23	**0.94**
Mokibelo	2009/07/27	**1.88**
Mokibelo	2009/07/29	**1.42**
Mokibelo	2009/08/03	**1.26**
Mokibelo	2009/08/05	**1.47**
Mokibelo	2009/08/07	**1.78**
Mokibelo	2009/08/10	**1.24**
Mokibelo	2009/08/12	**1.36**
Mokibelo	2009/08/14	**1.40**
Munyani	2009/05/05	**10.66**
Munyani	2009/05/12	**1.72**
Munyani	2009/05/18	**2.83**
Munyani	2009/05/25	**1.40**
Munyani	2009/05/27	**1.68**
Munyani	2009/06/02	**2.51**
Munyani	2009/06/08	**2.63**
Munyani	2009/06/09	**1.81**
Munyani	2009/06/16	**6.24**
Munyani	2009/06/23	**5.38**
Munyani	2009/07/02	**4.46**
Munyani	2009/07/06	**7.01**
Munyani	2009/07/08	**7.61**
Munyani	2009/07/14	**4.32**
Munyani	2009/07/20	**8.94**
Munyani	2009/07/23	**1.85**
Munyani	2009/07/27	**2.49**
Munyani	2009/07/29	**2.02**
Munyani	2009/08/03	**2.37**
Munyani	2009/08/05	**2.65**
Munyani	2009/08/07	**2.67**
Munyani	2009/08/10	**2.36**
Munyani	2009/08/12	**1.96**
Munyani	2009/08/14	**1.97**
Radimpe	2009/05/05	**2.91**
Radimpe	2009/05/13	**2.05**
Radimpe	2009/05/19	**2.82**
Radimpe	2009/05/25	**1.76**
Radimpe	2009/06/02	**1.40**
Radimpe	2009/06/15	**2.24**
Radimpe	2009/06/23	**1.69**
Radimpe	2009/07/01	**2.71**
Radimpe	2009/07/08	**1.95**
Radimpe	2009/07/14	**2.32**
Radimpe	2009/07/21	**1.72**
Radimpe	2009/07/28	**1.69**
Radimpe	2009/08/03	**1.99**
Radimpe	2009/08/06	**1.92**
Radimpe	2009/08/11	**1.88**
Radimpe	2009/08/13	**2.04**
Tharo	2009/05/14	**4.22**
Tharo	2009/05/20	**2.39**
Tharo	2009/05/29	**2.54**

Pedi	2009/08/13	**2.72**
Pedi	2009/08/13	**2.07**
Pedi	2009/08/13	**2.70**
Pedi	2009/08/13	**1.91**
Pedi	2009/08/13	**2.97**

Tharo	2009/06/22	**3.76**
Tharo	2009/07/06	**4.07**
Tharo	2009/07/22	**3.13**
Tharo	2009/07/29	**4.28**