DETERMINING THE REPRODUCTIVE STATUS OF 3 FEMALE FREE-RANGING WHITE RHINOCEROS (*CERATOTHERIUM SIMUM SIMUM*) BY MEASURING FAECAL PROGESTAGEN LEVELS

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ABSTRACT

There is still a lot unclear about the reproductive cycle of female white rhinoceroses. Limited information is available and the existing data are conflicting, especially regarding the oestrous cycle. This study was conducted from October 2008 until February 2009. In this period, three female free-ranging white rhinoceroses were monitored in the privately owned game reserve Lapalala. The aim was to collect as many faecal samples as possible in this period. The faeces were collected fresh and an Enzyme Immuno Assay (EIA) was performed to determine the progestagen levels in the samples. Reproductive progestagen levels were highly variable. The classification of Schwarzenberger *et al.* (1998) was used to categorize the animals according to their faecal progestagen levels. Due to the short study-period it is difficult to interpret the reproductive status of the three animals. It seems that measurement of the faecal progestagen levels is a reliable non-invasive way to collect information about the reproductive status of the animal. This is expected to become a valuable tool for the Lapalala-management and for conservation management in general.

INTRODUCTION

In total five rhinoceros species survive today; white rhinoceros (*Ceratotherium simum*), black rhinoceros (*Diceros bicornis*), Indian rhinoceros (*Rhinoceros unicornis*), Javan rhinoceros (*Rhinoceros sondaicus*) and Sumatran rhinoceros (*Dicerorhinus sumatrensis*). The black rhinoceros, Javan rhinoceros and Sumatran rhinoceros are critically endangered. Their populations are still decreasing. The status of the Indian rhinoceros is vulnerable, this population is recently increasing. The status of the white rhinoceros today is near threatened. The greatest threat for rhinoceros existence is poaching. Mostly, their horns are being used as an ingredient of traditional medicines in the Middle East they are also being used for ornamental use (Amin *et al.*, 2006).
In December 2007 there were an estimated 17.480 white rhinoceroses in the wild with a further 760 in captivity worldwide. The majority (98.8%) of white rhinoceroses occur in just four countries; South Africa, Namibia, Zimbabwe and Kenya. South Africa remains the stronghold for this subspecies (93.0%) conserving 16,255 individuals in 2007 (IUCN, 2008).
The Lapalala Wilderness Reserve has made it their mission to provide a breeding sanctuary for both the black and the white rhinoceros. At the last count (October 2008) 38 white rhinoceroses were identified in the Lapalala Wilderness.

From earlier research it seems that there is still a lot unclear about the reproductive cycle of female white rhinoceroses. Limited information is available and the existing data are conflicting, especially regarding the oestrous cycle. Earlier studies mostly were conducted in zoos, driven by the low rate of reproduction of white rhinoceroses in captivity. The dominating problem for the reduced rate of reproduction is absent or erratic oestrous cycle activity in over 50% of the females in the European and North American Species Survival Program (Hermes *et al.*, 2006).
Owen-Smith (1988) reported an oestrous cycle of approximately 30 days. This was based on only behavioural observations on free-ranged white rhinoceroses and not on endocrine data.
Schwarzenberger *et al.* (1998) classified rhinoceroses into four categories on the basis of oestrous cycle length and luteal phase 20-oxo-P levels: (1) regular oestrous cycles of 10 weeks duration and >800 ng/g faeces; (2) oestrous cycles between 4-10 weeks and 250-750 ng/g faeces; (3) no apparent cycle regularity, but luteal activity indicated by 100-200 ng/g faeces; (4) no apparent luteal activity as indicated by < 100 ng/g faeces. This led to the conclusion of the study; two thirds of the white rhinoceroses had erratic or missing luteal activity. Variable cycles of 4-10 weeks were found in six females and regular oestrous cycles of 10 weeks were found in only two females in this study.
Patton *et al.* (1999) found evidence of 17 reproductive cycles in five female white rhinoceroses. These cycles appeared to fall into two types; Type I with a cycle of approximately one month in duration and Type II with a cycle of approximately two months in duration. Interluteal phase lengths were similar for both cycle types but Type II cycles were characterized by extended luteal phases. Patton *et al.* (1999) diagnosed pyometra in two rhinoceroses that exhibited extended luteal phases (Type II) and uterine inflammation was diagnosed in an earlier study of a single white rhinoceros that exhibited extended luteal phase (Radcliffe *et al.*, 1997). Patton *et al.* (1999) hypothesized that some extended luteal phases are the result of uterine pathology and/or early embryonic death. Type I dominated in the data of this study and they suggested that Type II cycles might be aberrant.
In a study by Brown *et al.* (2001), they found that about half of the white rhinos (6 of 13) showed no evidence of ovarian cyclicity. In this study, 16 captive female black rhinoceroses

and 13 captive female white rhinoceroses from 14 different American institutions were monitored. Of the cycles observed, five were short (32.8 ± 1.2 days) and 24 were long (70.1 ± 1.6 days). The animals were tested in a period of 12-24 months and only two animals cycled continuously throughout the study.

As a result of these studies one can conclude that there is a huge variability regarding cyclicity in white rhinoceros females whilst in captivity, and kept under the current husbandry conditions. In addition, it seems that many captive white rhinoceroses do not cycle at all. Because of the reproductive problems in these animals, there is a very low birth-rate in captive rhinoceroses. So, to establish healthy self-sustaining populations of white rhinoceroses it is of great importance to understand the reproductive status and factors that influence the fecundity of these animals.
To ensure the best possible survival of rhinoceroses in Lapalala, management practice plays a crucial role. By collecting frequently faecal samples of three white rhinoceroses, their reproductive status can be determined by measuring faecal progestagen levels.
Monitoring reproductive hormones allows identification of pregnant, cycling and non-cycling females and the reproductive condition of males. For the staff at Lapalala this knowledge can possibly influence management procedures and the overall conservation management decision-making process.

MATERIALS AND METHODS

Animals and study site

Animals in this study included three female white rhinoceroses;

1. Tharo, 15.8 years old. Calf: Moruleng, 1.6 years old
2. Grikie, 19.8 years old. Calf: Diretse, 1.1 years old
3. Munyani, 12.7 years old. Calf: Mokebelo, 5.0 years old

They all live in Lapalala Wilderness, which is a large area of 36.000 hectares. They are all proven breeders of adult age and they all had a calf with them during the study-period. The selection of the animals took place during the first week of arrival in Lapalala. I wanted to monitor animals who live in different groups and in different areas of the reserve. There are a lot of variables which can influence reproduction but this way I could monitor cyclicity of cows in different social structures and in different environments.

There are two territorial bulls at Lapalala; Hatton and Poacher. Munyani and Tharo were Hatton's cows and Grikie was Poacher's cow. The females live in groups with other females and their offspring. The intercalving period is approximately three years (Bertschinger, 1994). Calves are weaned at about 12 months of age and usually stay with their mother for the first 3 years of their lives. Then they will leave and join other rhinoceroses (usually other sub-adults) and the mother will give birth to a new calf. The pregnancy duration is approximately 16 months in white rhinoceroses (Bertschinger, 1994). Because the intercalving period is three years I would expect the mother to start cycling again when the calf had reached a minimum age of 1.6 years old. Bulls live solitary but often I would find them with the group, inspecting the females.

White rhinoceroses have the habit of defecating in communal dung heaps. These heaps are usually created by the territorial bull in that area, which kicks his faeces around to spread it after defecating. Female animals use the heaps too but do not kick their faeces around. Often rhinoceroses defecate at the same time, especially mothers and calves (Owen-Smith, 1988; Pienaar, 1994). This and the use of communal heaps makes it sometimes difficult to assure sample identification. This is another reason why it is very important to actually observe the defecation of the specific animal. The rhinoceroses in this study all had an unique ear notch which I used to identify the animals.

I started my study in October 2008, and in that period the rhinoceroses were being fed by the management of Lapalala. Each morning they would get lucerne from the rangers, a supplement during the dry season to the natural vegetation which was generally inadequate for the rhinoceroses to feed on. The animals were being fed until the 7 November 2008, by that time the rains had come and everything greened up. There are multiple groups of rhinoceroses living together in Lapalala and each group had a specific feeding spot in the area they lived in. Until November, I would find the rhinos almost every morning at the feeding spot. There were several dung heaps near the feeding spots so it was not a problem to collect faeces during that period.

Sample collection

My aim was to collect as many fresh faecal samples as possible for the three female rhinos. To be sure about the identity of the animal, it had to be identified first by ear notch. Subsequently, I had to follow the animal and wait for it to defecate. This way I was absolutely sure about the identity and also that the faeces was as fresh as possible. Schwarzenberger *et al.* (1998) found that progesterone metabolite concentration did not differ significantly between the central part and the outer part of the faecal balls. Whole balls with the smallest

amount of insects were chosen for faecal collection. Undigested materials and parasites were avoided.
Collection of faeces was done manually with gloves (Hartmann Peha-soft, REF:942150). After collection in glass sample cups, samples were stored temporarily in a cool box as soon as possible, and back in the camp immediately frozen at -20°C until analysis.
Faecal samples were mostly collected in the early morning (between 6-8 a.m.) and in the afternoon (between 4-6 p.m.). On average I usually collected 1-2 samples of each animal per week. The total study-period in Lapalala was three months; from October 14, 2008 until January 20, 2009.

Second study Annemieke van der Goot
During the period when this study was conducted, there was another study ongoing in Lapalala too. My colleague Annemieke van der Goot also worked on reproductive research on an additional three female white rhinoceroses. In addition she did a pilot-study on the use of Very High Frequency- telemetry. For the use of this system, transponders had to be placed in the rhino horns and to do that the animals had to be immobilised. We were present when the animals were immobilised, which was led by the veterinarian. During the immobilisation blood samples were taken from the ear veins of the three animals. These samples were sent to Onderstepoort and pregnancy tests were done. Two of the three animals were pregnant according to these tests. The coat-a-count progesterone solid phase iodine-125 radioimmunoassay for the direct, quantitative measurement of progesterone in serum was performed on the blood samples. Progesterone levels in serum had to be interpreted by the guidelines given;

Not pregnant:	<4.5 nmol/l
3-4 months pregnant:	4.5-6 nmol/l
5-8 months pregnant:	7-50 nmol/l
9-12 months pregnant:	50-70 nmol/l
13-16 months pregnant:	70-90 (and greater) nmol/l

One of the animals, named Radimpe (result progesterone; 103.89 nmol/l blood) gave birth early December and the other one, named Motklaki (result progesterone; 57.00 nmol/l blood), was still pregnant when we left Lapalala. All the samples from Annemieke's study were extracted and analysed the same way as the samples in this study. Though it is not my research, it could still be useful to compare the progestagen levels between Motklaki and the animals monitored in my study (van der Goot, 2009).

Faecal extraction
The extraction and analysis of the faecal samples took place at the Veterinary Faculty Onderstepoort, of University Pretoria (South-Africa).
Firstly, dry frozen faeces was lyophilised for 29 hours (see photo 1). This was done by a machine (Instruvac Freeze-drier from Air & Vacuum Technologies, model: VFDT 02.50, manufactured and purchased in South-Africa) on a bulk tray and with a stopping system to reduce water content and water variability in the different samples. After freeze-drying the samples had to be pulverised. For pulverization two small sieves were used so that only small powder could fall trough (see photo 2). To avoid cross contamination the tweezers and sieves were soaked in 80% EtOH (prepared from Ethanol Absolute 99%, Merck, Saarchem, diluted with distilled water) in between the samples. After this process, the faecal samples were transformed into faecal powder. After the pulverization the samples had to be weighed; of each sample 0.50-0.55 gram was placed in a sample tube (Kimble Borosilicate Glass, Disposable Culture Tubes, 12x75mm) and the exact weight was noted. Final separation took place by adding 3 ml of 80% EtOH to each sample. This was then placed onto a multi-shaker

on high speed for 15 minutes. After shaking, the samples were placed in a centrifuge and centrifuged for 10 minutes at 3000 rpm. With a pipette 1.5-2.0 ml clear supernatant was removed from the tube after centrifuging and transferred to another tube. This was stored at -20°C and the next day the samples were ready for Enzyme Immuno Assay (EIA). For details, see attachment 1.

Enzyme Immuno Assay

Faecal extracts were analysed with an EIA (see photo 3). The plate was coated with primary antibodies, the antibodies were raised against 5β-pregnan-3α-ol-20-one. This has previously showed to provide reliable information on progestagen levels in rhinoceros (Schwarzenberger *et al.*, 1998; Graham *et al.*, 2001). 4-pregnene-3,20-dione (progestagen) was used as standard and 5α-pregnane-3β-ol-20-one-3HS:DADOO-B was used as label. The sensitivity of the assay at 90% binding was 0.3 pg per well and inter- and intra-assay coefficients of variation ranged between 6.4% and 10.5%.

In brief, 50 µL of progestagen-standard, buffer, quality controls and diluted faecal extracts were pipetted in duplicate into plate wells. Another 50 µL of biotin-labelled steroid and antibody-serum was added. The plates incubated overnight at a temperature of 4 ºC. The next day, the plates were washed four times and 150 µL of streptavidin-peroxidase was added to each well and the plate was shaken for 30 minutes. The reaction was terminated by adding 50 µL of H2SO4 and followed by reading of the absorbance at 450 nm. For details, see attachment 2.

Data analysis

The first step in determining the status of the female rhinoceroses is to determine whether there is an indication of a cycle in the pattern of faecal progestagen levels. Definition of the follicular (FP) and luteal phases (LP) of the reproductive cycle is based on faecal progestagen levels. During the luteal phase the corpus luteum is responsible for progestagen production, so it would be expected that the progestagen levels would be higher during the luteal phase compared to the follicular phase (Senger, 2003). Schwarzenberger *et al.* (1998) defined the onset of the luteal phase as the first point after the values had increased by > 50 ng/g faeces and remained at 120 ng/g faeces for at least two consecutive values. The end of the LP was defined as the first of two consecutive values that were less than 120 ng/g faeces.

With only three animals included in this study and with the influence of other variables it is unfortunately impossible to test for significant differences.

Photo 1. Total amount of samples collected (including Annemieke's samples) in a cooling box. Not yet processed.

Photo 2. Processing the fecal samples into fecal powder using two sieves.

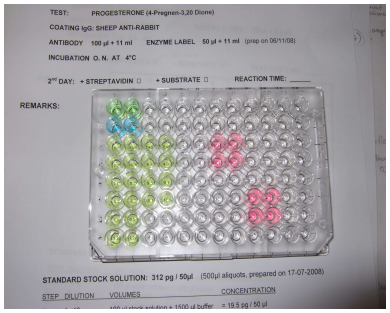


Photo 3. Example of an EIA.

RESULTS

Table 1 .Dates of sample collections and progestagen levels of animals included in the study.

Name	Date sample (dd/mm/yyyy)	Days	Prog. Con. (ng/g DW)
Tharo 1	20/10/2008	0	299.4
Tharo 2	28/10/2008	8	227.3
Tharo 3	04/11/2008	15	450.0
Tharo 4	04/11/2008	15	492.0
Tharo 5	07/11/2008	18	478.2
Tharo 6	10/11/2008	21	270.6
Tharo 7	23/11/2008	34	433.6
Tharo 8	09/12/2008	50	331.7
Tharo 9	20/12/2008	61	281.5
Tharo 10	03/01/2009	75	17.8
Grikie 1	27/10/2008	0	6.4
Grikie 2	30/10/2008	3	10.5
Grikie 3	05/11/2008	9	10.1
Grikie 4	18/11/2008	22	9.1
Grikie 5	26/11/2008	30	1.4
Grikie 6	08/12/2008	42	3.6
Grikie 7	22/12/2008	56	7.3
Grikie 8	05/01/2009	70	9.9
Grikie 9	15/01/2009	80	6.8
Munyani 1	03/11/2008	0	19.1
Munyani 2	08/11/2008	5	38.3
Munyani 3	11/11/2008	8	14.2
Munyani 4	23/11/2008	20	11.1
Munyani 5	01/12/2008	28	12.1
Munyani 6	10/12/2008	37	26.0
Munyani 7	15/12/2008	42	36.9
Munyani 8	20/12/2008	47	23.4
Munyani 9	23/12/2008	50	17.0
Munyani 10	06/01/2009	64	36.1
Munyani 11	12/01/2009	70	23.7
Munyani 12	14/01/2009	72	49.8

During this study, three female animals were monitored for 80 days. Unfortunately there is a wide variability between the days of sample collection. As a result there are gaps in time when progestagen levels are unknown. This makes it difficult to draw conclusions about the reproductive cycle in these animals.

Looking at the data of the three animals included in this study, it is noted that Tharo has much higher progestagen levels than the other two animals. Tharo has average progestagen levels of 328.21 ± 144.59 ng/g faeces. Unlike Tharo, Grikie has much lower levels, average progestagen levels of 7.23 ± 3.11 ng/g faeces. The third rhinoceros Munyani has average progestagen levels of 25.64 ± 12.14 ng/g faeces.

Schwarzenberger *et al.* (1998) classified the cycle characteristics into four categories:

1. Females with regular oestrous cycles of approximately 10 weeks and high luteal phase progestagen levels of >800 ng/g faeces.
2. Females with oestrous cycles ranging in length from 4-10 weeks and luteal phase progestagen levels of 250-750 ng/g faeces.
3. Females with no apparent oestrous cycle regularity but some luteal activity with progestagen levels of 100-200 ng/g faeces.
4. Females exhibiting no luteal activity with progestagen levels <100 ng/g faeces.

Because it is almost impossible to see an obvious complete cycle in the short period of 80 days, I will categorise the animals mostly on basis of their progestagen levels.

According to Schwarzenberger *et al.*, Tharo can be classified as a category 2 animal. Looking at her progestagen levels, there seems to be quite some variation (see figure 1). Even on the same day, day 15, there is a difference in faecal progestagen levels between the morning-sample and the afternoon-sample. It seems that even during the day the levels can fluctuate, although it does not change the category. Because the progestagen levels are this high, we can be sure that there is luteal activity. It is unknown when this progestagen increase has started but the levels seem to decrease from day 34. We cannot be sure about that because of the gaps in between. For example, there could be a drop between days 34-50 or there could be a peak. It makes a difference because the drop would probably mean the luteal phase has ended and a peak could be the start of a new cycle. But looking at the other levels a peak does not seem very likely. Another possibility is the fact that Tharo could be pregnant hence the high levels of progestagen. Motklaki (van der Goot, 2009) has average progestagen levels of 250.95 ng/g faeces and she is pregnant. When we compare the progestagen levels of Tharo and Motklaki it seems that they are in the same range. But on the last day a sample of Tharo was collected, there was a level of 17.8 ng/g faeces found. This indicates pregnancy is not very likely. During pregnancy, progestagen levels should be constantly high. There is still a possibility that Tharo was pregnant and miscarried or maybe there was a measurement error. It could be that the last level of 17.8 ng/g faeces is a wrong result from the EIA and that would mean Tharo is probably pregnant. Still, the most reasonable interpretation would be that Tharo is cycling and there is a luteal phase of at least 34 days because of the drop in progestagen on day 75. From day 34 on the progestagen levels are decreasing and might reach the baseline levels from day 75 and further. In addition, there have been no observations of Hatton following Tharo around and Moruleng is still young (1.6 years old). Unfortunately it is not possible to be sure because there are no data available.

Patton *et al.* (1999) recorded 2 types of cycles. One of approximately one month of duration (type I) and one of approximately two months of duration (type II). Evidence suggested that some type II cycles are aberrant and type I cycle is the typical reproductive cycle of female white rhinoceroses. One of the females included in that study displayed both type I and type II cycles. Knowing this, we cannot exclude that Tharo is not displaying a type II cycle. To be sure about the length of the cycle, more data have to be collected over a longer period. Also, this study was performed with a population of captive rhinoceroses instead of free-ranging animals. It is possible that the animals display Type II cycles only in captivity and not in the wild.

Looking at table 1 and figure 2, we can immediately see that there is a big difference. Grikie has, compared to Tharo, very low faecal progestagen levels. According to the categories she would be classified as a category 4 animal. There is no luteal activity because the progestagen levels are < 100 ng/g faeces. One conclusion is that Grikie was not pregnant during this study. Still, that does not mean she is not cycling. Because of the short period, it can be possible that her progestagen levels will increase after day 80 or that she just displayed a cycle before the first sample collection took place. Grikie has a young calf of 1.1 years old, so a possibility is a

lactation anoestrus. There is not much known about lactation anoestrus in rhinoceros. More research has to be done in that area. During the study-period there have been observations of Poacher (the bull) following Grikie around. This usually indicates that the cow is in oestrus (Owen-Smith, 1988), but looking at the progestagen levels that does not seem to be the case. Grikie has already had five calves so she must have been cycling in the past. Yet that does not mean she is still cycling. Another explanation could be that she is in the follicular phase. To be sure about that, one has to measure the estradiol levels. Unfortunately, the non-invasive way to measure estradiol levels would be by collecting urine over a specific period. The collection of fresh urine is practically and logistically almost impossible in free-ranging animals, so that was not an additionally option in this study.
Munyani can also be classified as a category 4 animal (see figure 3). Her faecal progestagen levels are comparable with Grikie's, only Munyani's levels are slightly higher. There seems to be no luteal activity, because the levels are very low. In addition we can conclude that she too is not pregnant. The last sample however could be the start of a luteal phase because from day 70 there is a big increase in progestagen levels visible. On the other hand, the progestagen levels seem to be very unpredictable judging the other levels. So again, we cannot be sure about that. The remarkable thing about Munyani is that she still has a calf older than five years with her. The normal intercalving period of white rhinoceroses is approximately three years so after five years you would have expected her to conceive again. Because Munyani already had a calf, she is a proven breeder. Most of the problems concerning reproduction in captive rhinos are based on the fact that nulliparous animals won't reproduce. So the question that can be raised is; why hasn't Munyani calved again? It could be that she does not cycle at all, but since she already had a calf that is not a likely possibility. There could be a pathological reason for the acyclicity, like a reproductive disorder or disease. In order to exclude that Munyani would need a health-check. To exclude pathologic reasons for reproductive failure, blood needs to be tested for hormone-concentrations and ideally ultrasound examination has to be performed.

Figure 1. Faecal progestagen levels

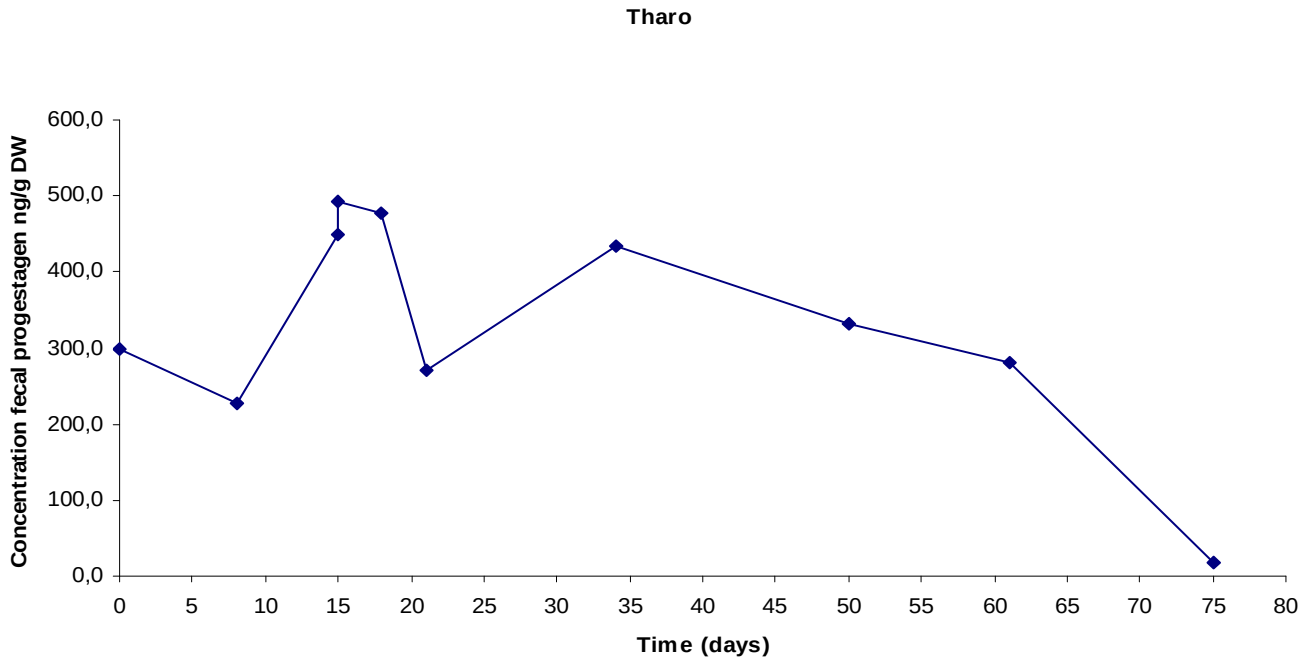


Figure 2. Faecal progestagen levels

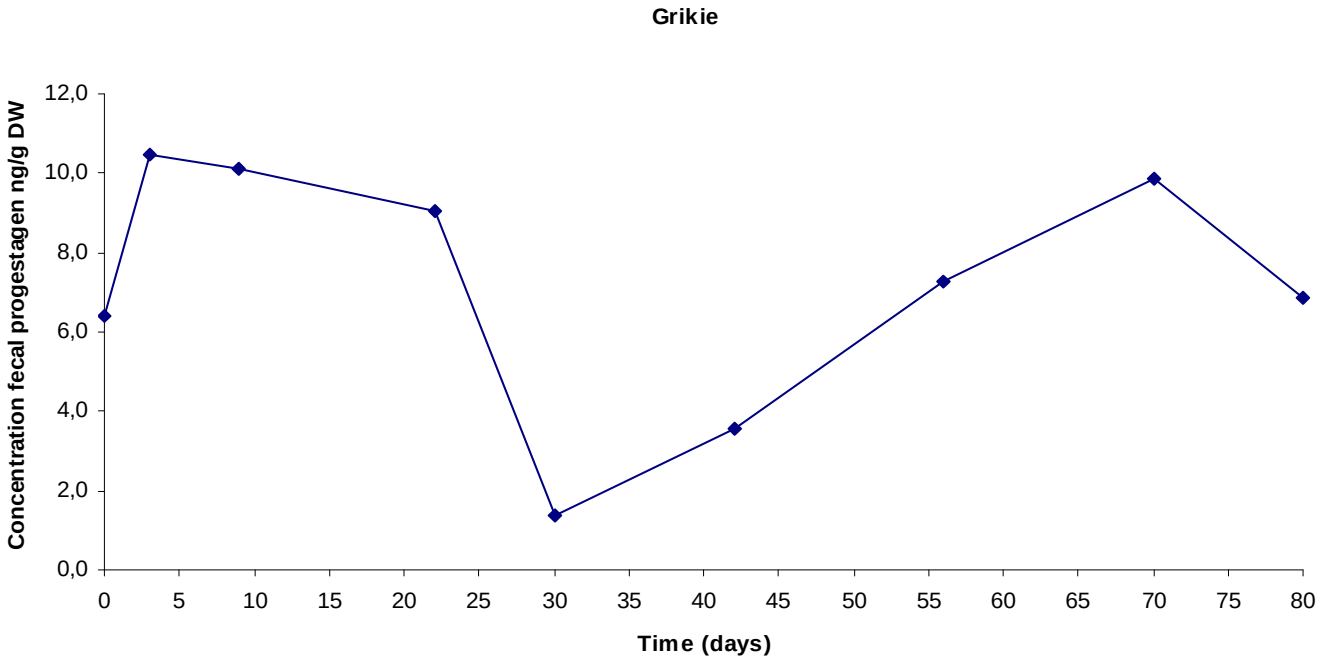
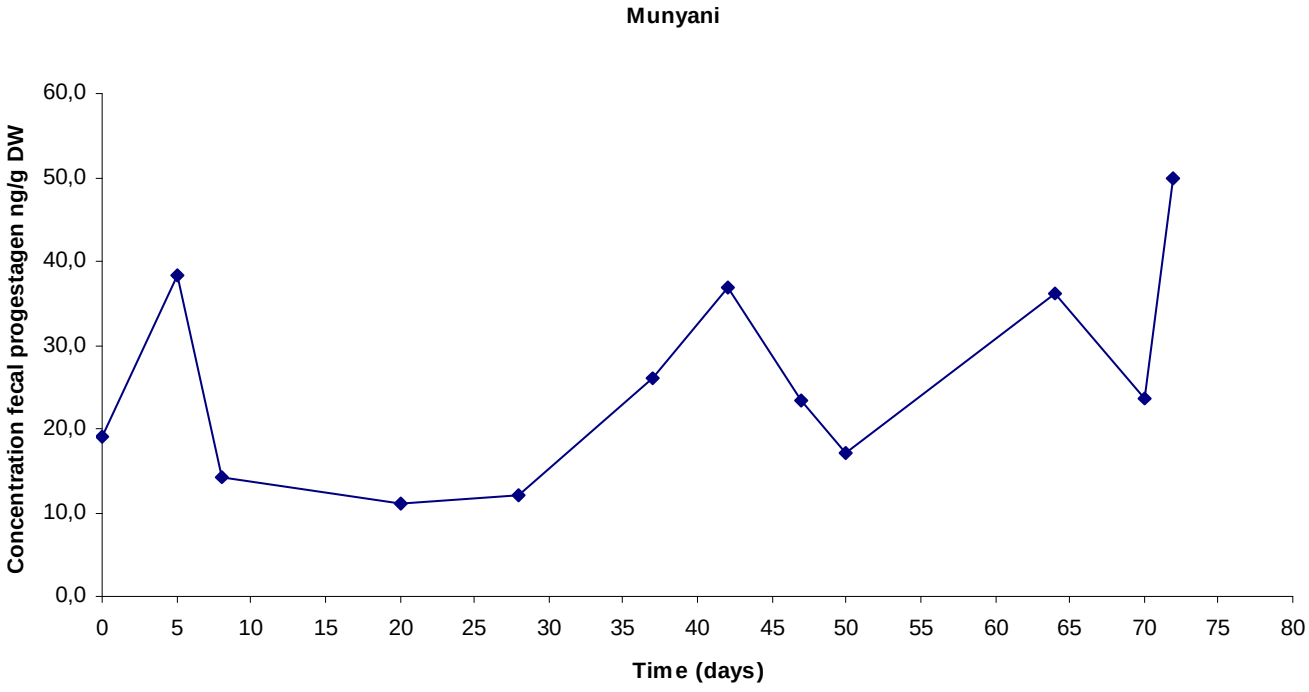


Figure 3. Faecal progestagen levels



DISCUSSION

The aim of this study was to learn more about the reproductive status of three female white rhinoceroses in Lapalala Wilderness. I was one of the two students who started this IBREAM project at Lapalala which is a long-term study, to be carried out by different students. In order to be sure about the conclusions regarding the reproductive cycle of female white rhinoceroses, more data needs to be collected over a longer period.
Despite earlier research it seems that it is still very difficult to make generalisations about the reproductive cycle in white rhinoceroses. I have only monitored the animals for three months and there was a wide variability in sample collections. It is possible that there was a peak of drop in the progestagen concentration during a period which could have been missed. This could change de categories in which the animals were classified. Sometimes it was very difficult to find the animals to begin with; there were weeks when I could only collect two samples in total. We found the animals with the help of an experienced guide. He was able to find the animals by tracking the footprints. Bad weather and some types of soil can make it difficult to find and follow footprints, so we could not find the animals every single day.
Because there was not a regular period in between the sample collections, there are gaps regarding the progestagen levels. Unfortunately, this makes it very difficult to draw conclusions about the reproductive cycles. In order to overcome this problem the frequency of sample collections needs to be higher. This could be achieved by monitoring less rhinoceroses (one instead of three). By monitoring only one animal you can collect more samples in the same time period but then the significance would also be less. Another way to collect more samples is by increasing the manpower. This way, you can collect samples from multiple animals and collect more samples per animal because there are more people available. In addition, more equipment (cars) would probably also influence the total sample amount positively. The advice depends on the overall aim of the IBREAM-projects and the financial situation.

In this report I mostly made my assumptions based on literature by Schwarzenberger *et al.* (1998). I categorized the animals into the four categories they have created. Patton *et al.* (1999), on the other hand had different results regarding cycle-types. They found only two types of cycles. Conflicting literature shows that there is a need to fully understand the reproduction in white rhinoceroses.
One of the main reasons why research on rhinoceroses is interesting is the fact that they do not reproduce well in captivity. In contrast, in the wild there does not seem to be a problem regarding reproduction in the white rhinoceros populations. Most of the time the females have a young calf with them or they are pregnant. This suggests that there are a lot of variables which influence reproduction in these animals. That is why it would also be very interesting to do research on climate-influence on reproduction. Is it for example important for rhinoceroses to experience a wet and a dry season? White rhinoceroses are spontaneous ovulators but does a long period of drought or rainfall influence their cyclicity? Also the partner choice seems to be very important and would be an interesting subject for future research.
Hermes *et al.* (2006) found that 56% of their studied population of nulliparous females had various reproductive pathology. Cystic endometrial hyperplasia, leiomyomas of the cervix, uterus and ovary, adenoma, cysts, and hydromucometra represent the scope of lesions identified. The stages of the lesions correlated with age. However, in parous females the incidence of reproductive lesions was significantly lower. In addition, 78% of these females

studied had erratic or absent luteal activity. According to the authors, this suggests the lack of ovarian activity by reproductive mid-life in non-reproducing females. The active ovarian status group had an age range of 3-19 years and the inactive ovarian status group had an age range of 15-28 years. There seems to be a range in which the ovaries of non-reproducing females start progressing towards an irreversible acyclicity with no follicular activity.
This study shows that there is a big problem regarding genital health in rhinoceroses. Especially nulliparous animals seem to be at risk. By reproduction in these animals, the incidence of pathology greatly minimises. Fortunately, most of the free-ranged rhinoceroses are able to reproduce. Still, there is a possibility that reproductive pathology can occur, especially between a certain age range. We should keep that in mind when we monitor free-ranging animals, like Munyani, who does not seem to be reproductive active.
Carlstead and Brown (2005) did research on the relationship between patterns of faecal corticoid excretion and behaviour, reproduction and environmental factors. They concluded that higher corticoid variability was found in noncycling as compared to cycling white rhinoceros females. This high corticoid variability appeared to be an indicator of chronic or bad stress and reproductive acyclicity. Social stressors may cause this chronic stress. In short, when dealing with a disappointing reproduction in a population, one has to consider the environmental factors and especially social factors. This could also be the case in a game park. The addition of new animals to a population or the removal of known animals can result in increased stress in the existing population and a reduced reproduction.

CONCLUSIONS

1. The measurement of faecal progestagen is a good non-invasive method to get information about the reproductive status of the free-ranging female white rhinoceros.
2. To be able to make assumptions and to draw conclusions, animals have to be studied over a longer period then 80 days and sample frequency has to be higher.
3. To statistically support conclusions, the amount of animals (*n)* included in the study needs to be higher. Then it is possible to check whether the progestagen concentrations are significantly different between the individual animals.
4. If this study can be carried out for a longer period, it has to potential to be very valuable for the management of Lapalala and for conservation management in general.
5. Despite some vagueness about the reproductive cycle in female white rhinoceroses this study can contribute to the development of a needed database.

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ATTACHMENTS

1.

STANDARD OPERATING PROCEDURE

Extraction Method for Dry Faecal Samples

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.

2.

STANDARD OPERATING PROCEDURE

ENZYME IMMUNOASSAY ON MICROTITRE PLATES USING BIOTINYLATED STEROIDS AS LABELS (Dr. Andre Ganswindt)

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°C)*
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°C)*
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°C)*
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°C)*
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°C)*
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. S**treptavidin 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. S**treptavidin 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. S**treptavidin 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.

4.10. Dispense 50µℓ of the stop reagent (buffer 1.11.) into each well. The blue colour turns yellow.

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

STANDARD OPERATING PROCEDURE

Extraction Method for faecal samples

1. 0.5 g wet faecal sample.
2. Add 1 mℓ dH_2O.
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_2O.
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. Vortex and let stand for 20 minutes. *Take note:* The whole pellet does not dissolve.
15. Dilute samples in assay buffer (buffer 1.3.) if required.

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