Non-invasive reproductive monitoring of six wild female white rhinoceroses (*Ceratotherium simum simum*)

Measurement of fecal progestagen levels as a part of a long-term study

Drs. B. Bitter

Gaining knowledge of the reproductive status of wild animals can provide invaluable information that may assist in making appropriate conservation management programs. This study was a part of a long term non-invasive reproductive study in wild southern white rhinoceroses (C*eratotherium simum simum*) females in the Lapalala Wilderness in South Africa.

Six females were monitored non-invasively using fecal progestagen analysis during a 4 months period. The purpose of this study was to determine the oestrus cycle of wild female white rhinoceroses and to non-invasively determine pregnancy. Fresh fecal samples were attempted to be collected on a regular basis (ideally twice per week) from each of the six females. The samples were freeze-dried and extracted and an enzyme immunoassay (EIA) was performed.

The fecal progestagen profiles were highly variable between the different females. Because of the difficulty of finding the rhinoceroses on a regular basis it proved to be difficult to determine the oestrus cycles of these females. However, the progestagen profiles showed a significant difference between pregnant and non-pregnant periods. Non-invasive measurement of progestagen levels could therefore be implicated in wildlife management for determining pregnancy in wild female white rhinoceroses.

Keywords: white rhinoceros, C*eratotherium simum*, reproduction, pregnancy, progestagen

Correspondence to: drs. B. Bitter, b.bitter@students.uu.nl
Utrecht University, Faculty of Veterinary Medicine, Department of Equine Sciences, the Netherlands

Project engaged to the Institute for Breeding Rare and Endangered African Mammals (IBREAM)
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Supervised by: dr. M. Paris, dr. J. Hanks & dr. F. Dalerum

Introduction

The Rhinocerotidae appeared some 50 million years ago. Today there are five rhinoceros species left; the black (*Diceros bicornis*), white (*Ceratotherium simum*),Indian (*Rhinoceros unicornis*), Javan (*Rhinoceros sondaicus*) and Sumatran rhinoceros (*Dicerorhinus Sumatrensis*). All five rhino species are endangered; they are all exposed to a permanent threat of poaching [IUCN 2008, Metrione L.C. 2007].

The black and the white rhinoceroses formerly occurred widely in the southern parts of Africa. From the seventeenth century onwards the European settlement of southern Africa increased and the black and white rhinoceroses were gradually eliminated from parts of their home range. Rhino horn is valuable for its supposed medicinal and aphrodisiac properties and for ornamental use especially in south-east Asia [Skinner J.D. et al 2003].

The white rhinoceros is the most common of all rhino species and consists of two genetically distinct subspecies; the northern (*Ceratotherium simum cottoni*) and the southern (*Ceratotherium simum simum*) white rhino. The northern subspecies has believed to gone extinct recently [Timesonline, International Rhino Foundation]. The southern subspecies has a population of over 15,000 individuals, but was at the end of the nineteenth century near extinction, with approximately 20 free-ranging individuals alive [IUCN 2008]. Presently almost all free-ranging southern white rhinoceroses are confined to the Republic of South Africa. The white rhinoceros is classified as “Near-Threatened” on the IUCN’s Red List of Threatened Species [IUCN 2008].

Conservation of the white rhinoceros is of great importance and to establish self-sustaining populations would require optimum breeding performances. Unfortunately the reproductive rate of captive rhinoceroses is very poor and especially the high incidence of long anoestrus periods, observed in captivity, is troubling [Hermes R. et al 2006&2007, Brown J.L. et al 2001]. There is few data on reproduction of white rhinos and most of the reproductive research has been conducted in captive environments. The data that do exist are furthermore conflicting. For example, it has been suggested that in captivity some female white rhinos have short cycles (31-35 days), some long cycles (73-78 days) and some do not cycle at all [Brown J.L. et al 2001]. The differences in duration between the short and long cycles could be explained by a difference in the persistency of the corpora lutea which leads to extended periods of elevated progesterone levels (prolonged luteal phase) [Schwarzenberger et al 1998]. Stress, disturbed social behaviour, nutrition, age and specific ailments may also influence the oestrus cycle [Carlstead K. and Brown J.L. 2005, Schwarzenberger F. et al 1998, Patton M.L. et al 1999, Owen Smith R.N. 1988, Hermes R. et al 2006]. There are more different cycle lengths recorded for female white rhinoceroses. Patton et al [1999] described the existence of a type I and type II cycle. He defined a type I as a cycle length of approximately 35 days and a type II cycle as a cycle with a duration of approximately 65 days. The type I cycle is presumed to represent the typical oestrus cycle for the white rhinoceroses. In captive animals studied by Schwarzenberger et al [1998] four different cycle categories were described: type 1:

estrus cycles of 10 weeks with high luteal activity, type 2: cycles of 4-10 weeks, type 3: with no estrus cycle regularity, but some luteal activity and type 4: with no luteal activity.

Sampling of fecal progestagen has become a useful way to assess reproduction in captive and wild rhinoceroses [Graham L. et al 2001, Schwarzenberger F. 1998, Berkely E.V. et al 1997, Garnier J.N. et al 2002]. Radcliffe et al [1997] reported that progestagen concentrations increased 7-9 days post ovulation and remained elevated for 19-22 days before returning to baseline values. Progestagen concentrations also remained high after conception and did not decrease until parturition. Ovulation occurred during lowest concentrations of fecal progestin. Progesterone levels increase after ovulation and remain elevated during pregnancy. Progesterone levels thus describe ovarian productivity of the white rhinoceroses [Senger P.L. 2003].

Since there is few data on the oestrus cycle of especially free-ranging white rhinoceroses, the aim of this study is therefore to non-invasively determine the oestrus cycle of six mature female white rhinoceroses living in a game reserve in South Africa. Although kept within this game reserve, they can be considered to be free ranging wild individuals as the reserve is larger then their home ranges. With the use of regular samples collected in the wild, my aim was to compare the oestrus cycles of these females with data published in the literature from animals that were kept in captivity. Understandability of the oestrus cycle of wild southern white rhinoceroses may enhance the reproduction in captivity of southern and northern white rhinoceroses. In this study the fecal progestagen levels were also measured to see if we can predict pregnancy using non-invasive measurement techniques, as this would provide a tool to be used for wildlife management.

Material and Methods

Study site and animals

The research was conducted in the Lapalala Wilderness game reserve. Lapalala covers approximately 36,000 ha, it has 88 km of pristine river frontage and is part of the Unesco Waterberg Biosphere. It is one of the largest privately owned game reserves in South Africa and they successfully breed white and black rhinoceroses. Recently the reserve is no longer open to tourists and the animals live freely with minimal human interaction. 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].

There are 44 white rhinoceroses living in Lapalala. I studied six white rhinoceros females (table 1) which were located in different sections of the park. The animals were selected based on ease to find them or identify them. One of the animals (Munyani) was hand-reared and thereafter replaced in the game reserve. Five of the females were presumed to be reproductively active (they reproduced in the past) and one animal (Mokibelo) was supposed to get reproductively active based on her age (white rhinoceroses start to show follicular activity at an age of three to four years) [Hermes R. et al 2006, Owen Smith R.N. 1988]. During the study two females calved and the probable date of births were noted. Two of the study animals had radio transmitters placed in their horn, but both of them failed working before the start of this study.

Reproductive monitoring included the collection of hormonal data for the six females mentioned during a four months period from the twelfth of January until the first of May.

Table 1. Information on the six studied females white rhinoceroses in the Lapalala Wilderness game reserve included in the current study. The days post partum (pp) describe the days pp at the beginning of my study period. Munyani lost her youngest calf shortly after birth; the date of parturition is unknown, but estimated to be 2,5 years ago.

Female ID	Date of birth	No. of calves	Birth date youngest calf	Mean interval births	Days post partum
Grikie	01-01-1989	5	18-08-2007	3,5 years	512
Mohlaki	01-12-1993	4	04-03-2009	2,7 years	694
Mokebelo	04-11-2003	/	/	/	/
Munyani	01-02-1996	2	unknown	/	± 500
Radimpe	01-04-1995	3	10-12-2008	2,5 years	33
Tharo	01-01-1993	3	27-03-2009	2,5 years	705

Fecal sample collection

The animals were tracked with a local game guide twice daily, early in the morning just after dawn and in the afternoon. White rhinoceroses show bimodal activity patterns with main active periods early in the morning and late in the afternoon, although this may shift on cloudy days and towards the dry season [Owen Smith R.N. 1988].

The animals were either tracked by car or by foot. Finding, identifying and tracking an individual rhino consisted of searching for its characteristic footprint (and that of the calve if the female was followed by one) on the roads in their home range. Each individual rhinoceros was characterized by the size and markings of its footprint, as well as by the features of its calf's footprint [Garnier J.N. 1998]. We identified the animals by sighting using their exterior marks, i.e. earnotches and size and shape of horn. We always made serious effort not to disturb the animals by staying downwind and by prevent making any noise.

Once the animals were found, either by direct sighting from the car or by following their tracks, they were positively identified by me and/or the field guide. Fresh fecal samples were collected from their tracks (before of after identification of the animal) or when defecation was seen. The fecal samples were collected from the ground. A sample was

considered fresh if the pellet was still wet and if no insect contamination had occurred. All samples with these characteristics were regarded to have been collected within an hour of defecation. Fecal samples were collected by breaking apart a fresh fecal pellet and collecting 10-40 gram of feces from the inner parts of the balls thereby avoiding insect contamination and removing most of the indigested material as possible. Gloves were used (Hartmann Peha-soft, REF:942150) and glass containers to collect and store the samples. After collecting the samples, the animal, date and time were noted on the container. The time difference between collecting the sample and positively identifying the animal and if defecation was seen was also noted.

The samples were stored in glass containers and put in a cooler box until stored in a permanent freezer at – 18^{o}C which occurred between 30 minutes and 5 hours with an average time of 1 hour and 37 minutes after sample collection.

Fecal sample extraction

The samples were transported frozen to the laboratory in Pretoria (University of Pretoria, Faculty of Veterinary Medicine, Onderstepoort) in a cooler box. There the samples were freeze dried for 48 hours in a vacuum oven (Instruvac ®Model: VFDT 02.50) at - 50^{o}C and 80-90 mTorr. The freeze drying is necessary in order to get all the liquid out of the samples to prevent variability in weight due to liquid in the samples.

The samples were pulverized using a small kitchen strainer and a set of tweezers to separate the dry material 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. The resulting dry powder was put back in the glass containers and stored under room temperature until extraction. Approximately 50 mg (in between 50 and 60 mg) was weighed out of every sample and put over 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 ml of freshly prepared 80% EtOH was added to each sample tube and all the tubes were vortexed on high speed for fifteen minutes. Thereafter the tubes were centrifuged on 3300 rpm for ten minutes. The supernatant was transferred into an Eppendorf tube and was stored at -20^{o}C, until final analysis.

Hormonal analysis by enzyme immunoassay

Fecal progestagens were measured with an enzyme immunoassay (EIA), previously described and validated for wild black and white rhinoceroses [Schwarzenberger F. et al 1998].

We used the protocol as described previously by Schwarzenberger F. et al [1996]. Briefly, the fecal progestagens were measured with an EIA that used an 4-pregnene-3,20-dione (progesterone) as standard. The EIA`s were performed in microtitre plates coated with primary antibodies, using a double antibody technique. The antibodies were raised

against 5beta-pregnan-3alpha-ol-20-one. The label used was 5alpha-pregnane-3beta-ol-20-one-3HS:DADOO-B.

The sensitivity (the proportion of true positives identified by the test as positive [Petrie A. et al 1999]) of the assay at 90% binding was 0,3 pg per well and the inter- and intra-assay coefficients of variation ranged between 6.4% and 30.5%.

For further details regarding the assay please see appendix A.

Data analysis

Data are presented as mean ± SD. A two sample t-test (unequal variances) was used to examine the differences between the pregnant fecal progestagen concentrations and the non-pregnant luteal phase progestagen concentrations. The difference between pregnancy hormone concentrations and the non-pregnant luteal phase hormone concentrations is based on the average of the pregnant two rhinoceroses during pregnancy and the average of the hormone peak levels of the non-pregnant, reproductively active, three rhinoceroses. Days of the sample collection describe the entire research starting 01-10-2008 until 01-05-2009. Days of the hormone profiles describe my study period starting 21-01-2009 until 01-05-2009. Fecal progestagen concentrations are noted as ng/g dry weight (DW).

Unfortunately the data collected from 01-10-2008 until 20-01-2009 can not be compared to the data I collected in my study period due to a shift in the linear range of the assay between these two sample batches. I will therefore consider the conclusions drawn from the data previously collected by Goot A.C. van der [2009] and Charbon Y.N. [2009] on the five communal female rhinoceroses, to determine whether my findings strengthen the initial assumptions made on each specific individual, based on their collected data set.

Results

Data collection

This study was conducted on free-ranging animals. It therefore proved difficult to find the animals weekly and to get weekly samples from all the animals (fig. 1). Based on the number of samples I managed to collect during my study period I can only make assumptions regarding the oestrus cycles of these six female rhinoceroses.

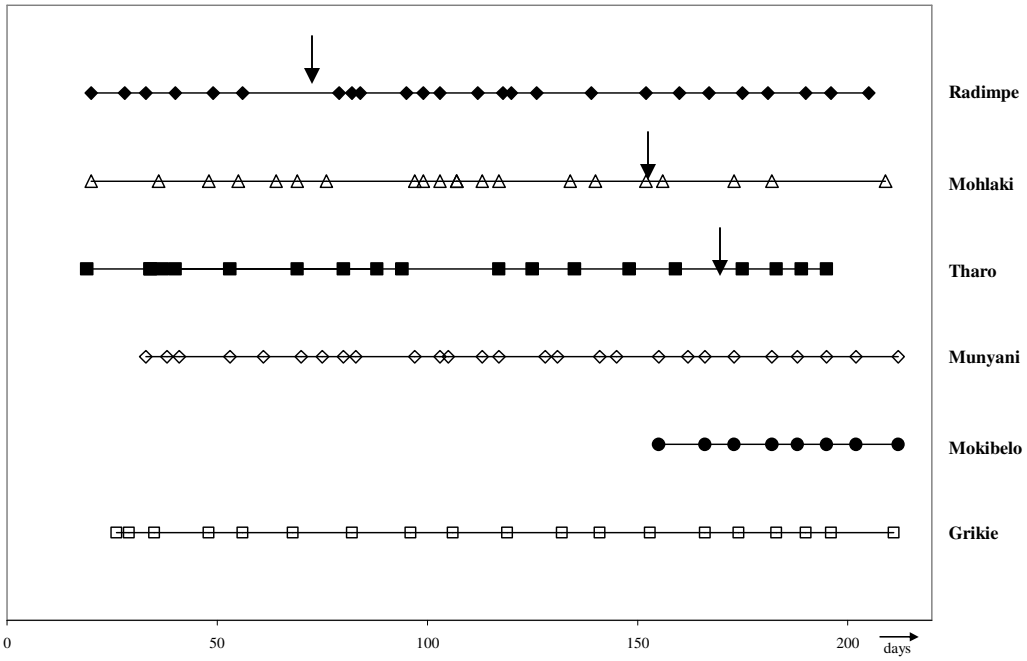

Fig. 1 Sample collection during the entire research. Day 01-10-2008 until day 01-05-2009
The average of days in between sample collections during 21-01-2009 until 01-05-2009 is; Radimpe: 7,15 (SD± 3,14), Mohlaki: 10,67 (SD± 8,13) , Tharo 8,78 (SD±: 4,16), Munyani: 6,60 (SD± 2,69) Mokibelo: 7,13 (SD± 2,16) , Grikie: 9,20 (SD± 3,30).
Arrows indicate date of parturition; Radimpe: 10-12-2008, day 70, Mohlaki: 04-03-2009, day 154, Tharo 28-03-2009, day 178.

Individual hormone profiles

The four profiles of the assumed non-pregnant females in this study all showed peak levels but most of them were not different from the mean hormone levels ± the standard deviation (SD). They showed nadirs six to eight days preceding the peak levels. Only Munyani and Grikie showed two peak levels that were different from the measured mean hormone concentrations. Radimpe`s profile (fig 2) showed one peak hormone level (41,4 ng/g DW with a mean progestagen concentration of 25,3 SD± 9,0). This peak hormone level was preceded by a nadir (10,7 ng/g DW). The hormone levels increased for the following six days at least. Grikies profile (fig 3) showed very low progestagen levels (mean progestagen concentration 17,0 SD± 5,4), lower than those of the other rhinoceroses measured. It also showed two peak hormone levels with approximately 70 days in between each peak. The hormone level at the highest peak (24,1 ng/g DW) was preceded by a nadir (7,2 ng/g DW). Munyani`s profile (fig 4, mean progestagen concentration 68,8 SD± 36,1) showed two peaks with 37 days in between. The highest peak level (166,70 ng/g DW) was preceded by a nadir (39,3 ng/g DW). The hormone levels thereafter increased following the next nine days at least. Mokibelo`s profile (fig 5, mean 40,1 SD± 19,0) was of a very short time duration which made it even more difficult to state anything about this profile. Though there seemed to be two peak hormone levels with 29 days in between, there was no nadir before these peaks.

Mohlaki`s hormone profile (fig 7) showed some fluctuation before parturition. The hormone levels two days after parturition (16,2 ng/g DW) were 1,14% of the levels two days before parturition (1424,7 ng/g DW). There is a gap in sampling after parturition, which makes it a bit unreliable to state anything further about the post partum hormone levels. Tharo`s hormone profile (fig 6) showed a low value likely due to a fault in sampling or analysis of the sample. The hormone value on day 24 is to low to be possible, since Tharo was certainly still pregnant at that moment. The date of parturition also does not match the obtained hormone data, hormone levels dropped already nineteen days before the estimated parturition. The day of parturition was based on visual findings of the animal with and without a calve.

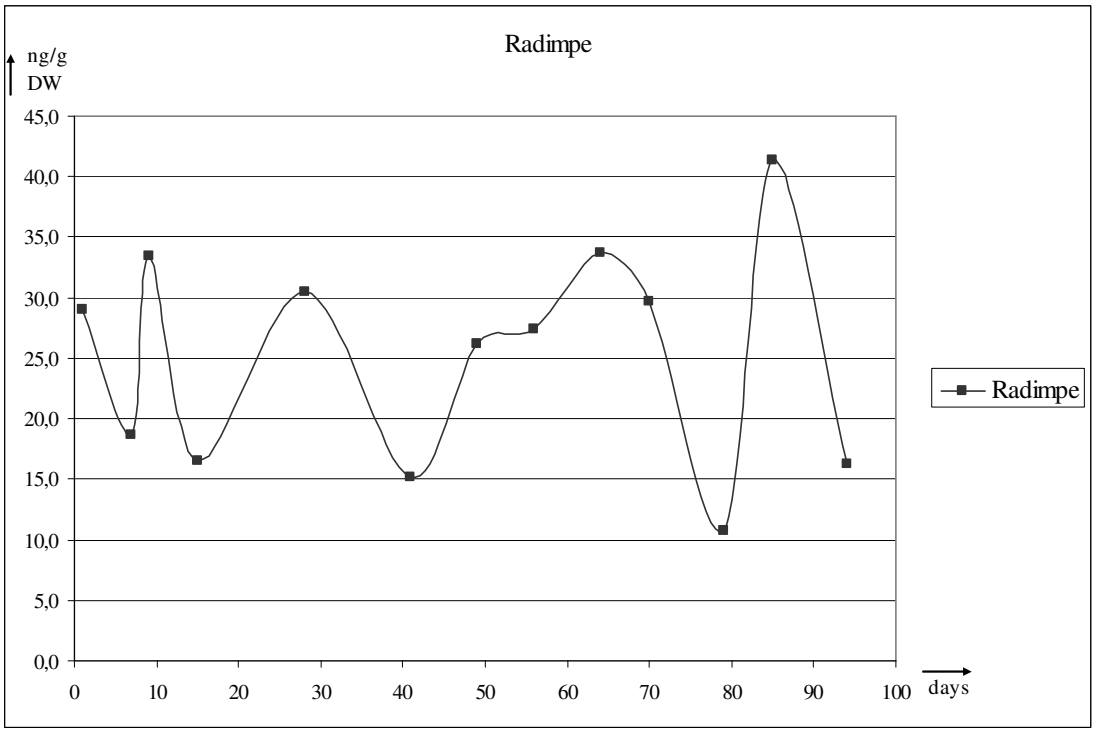

Fig 2. Fecal progestagen (ng/g dry weight) profile of Radimpe in days. Collected from 21/01/2009 – 24/04/2009

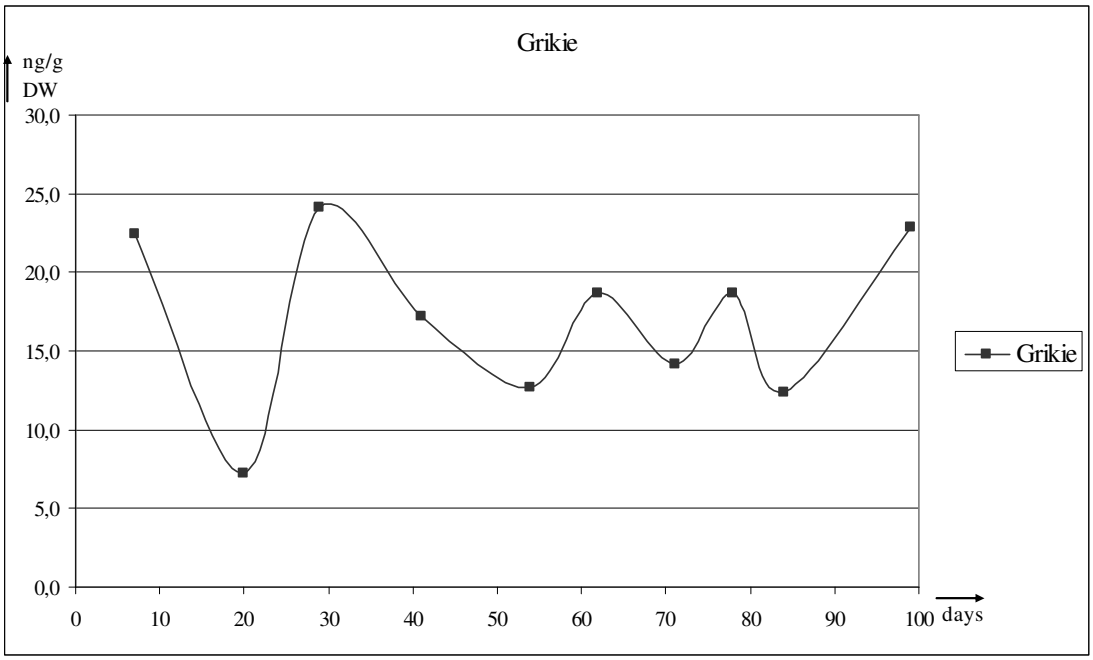

Fig. 3 Fecal progestagen (ng/g dry weight) profile of Grikie in days. Collected from 21/01/2009 – 30/04/2009

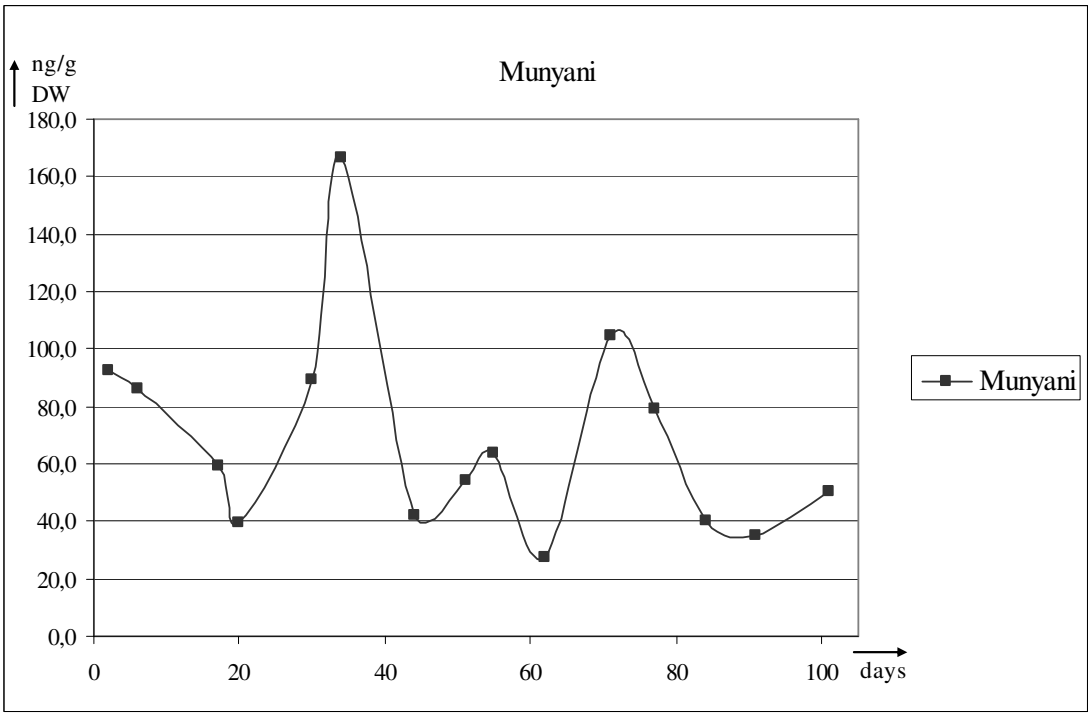

Fig. 4 Fecal progestagen (ng/g dry weight) profile of Munyani in days. Collected from 21/01/2009 – 01/05/2009

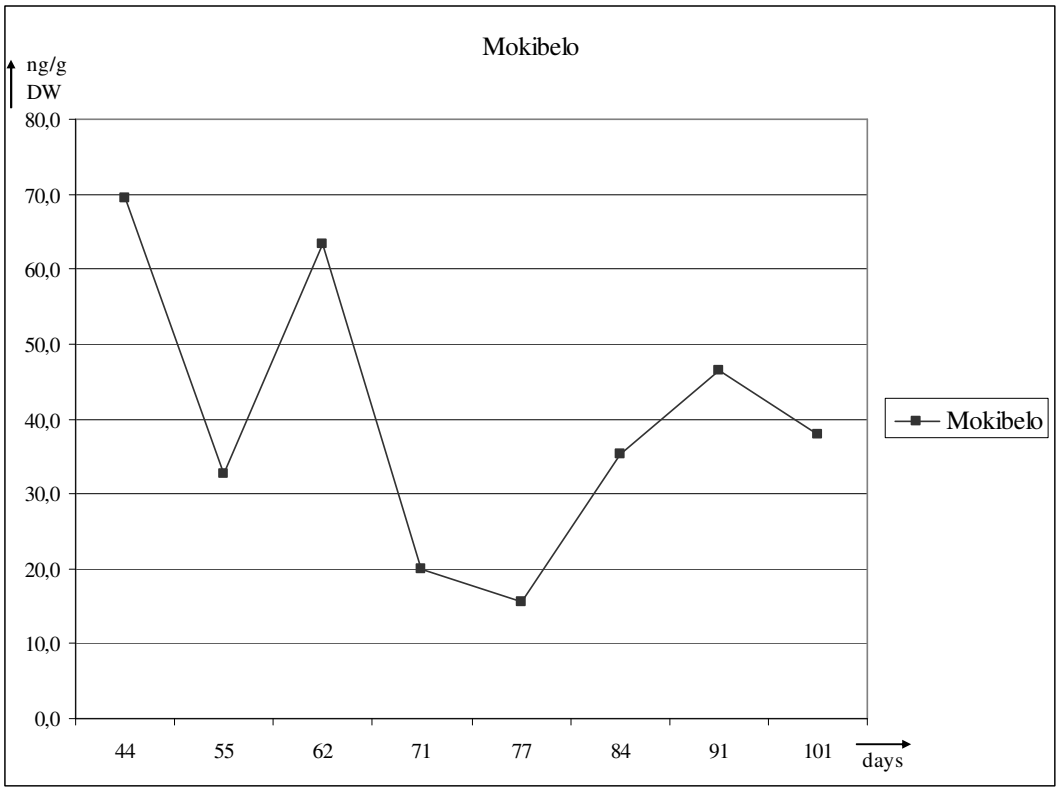

Fig. 5 Fecal progestagen (ng/g dry weight) profile of Mokibelo in days. Collected from 21/01/2009 – 01/05/2009

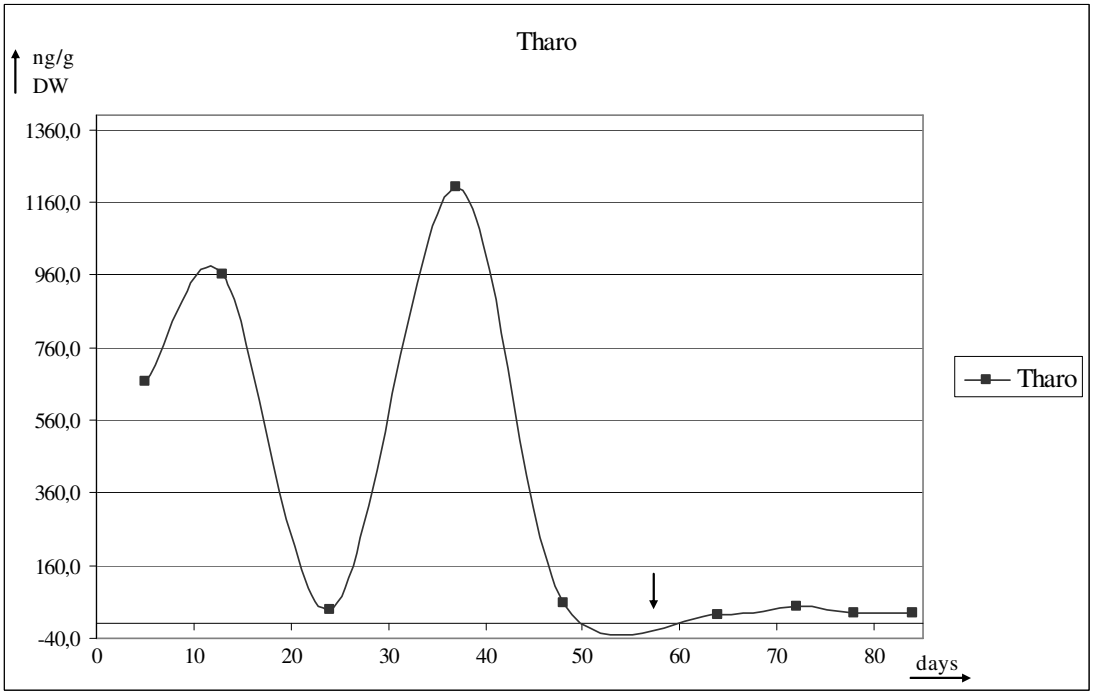

Fig. 6 Fecal progestagen (ng/g dry weight) profile of Tharo in days. Collected from 21/01/2009 – 14/04/2009. The arrow indicates parturition (28-03-2009).

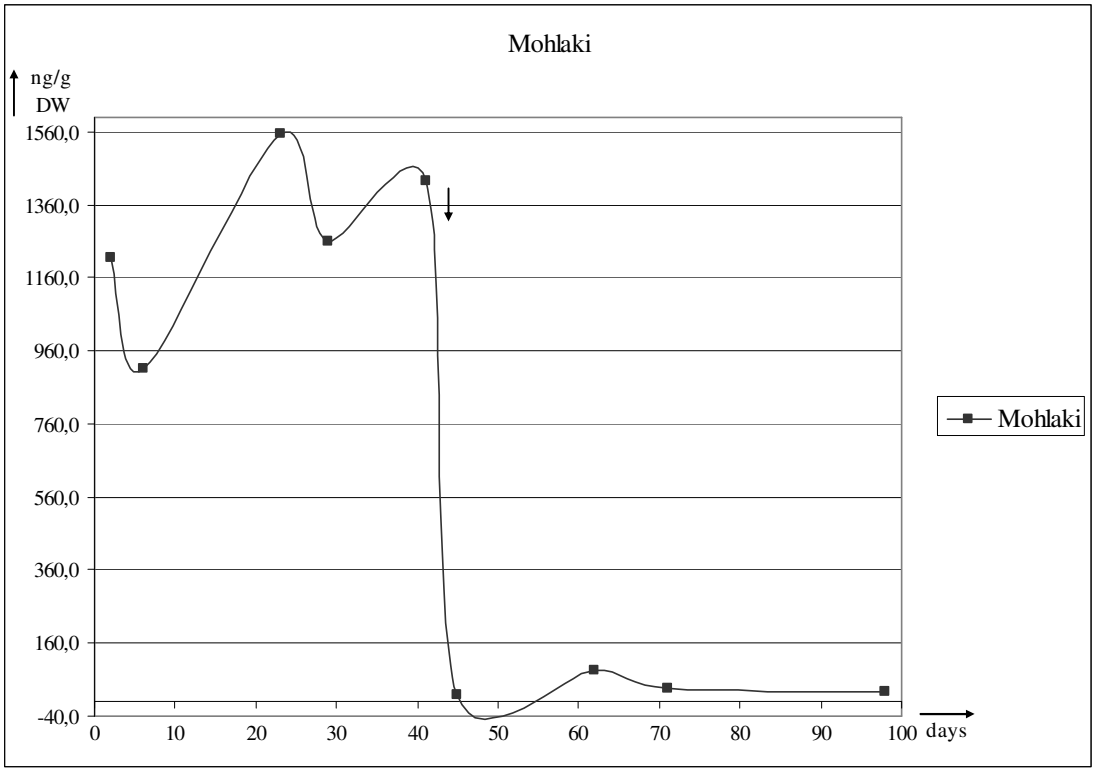

Fig. 7 Fecal progestagen (ng/g dry weight) profile of Mohlaki in days. Collected from 21/01/2009 – 28/04/2009. The arrow indicates parturition (04-03-2009).

Pregnancy

The hormone profiles showed a lot of diversity in between the individual females. This was partly due to the fact that two of the animals were pregnant and gave birth during the sample collection, whereas the other four animals were (presumably) non-pregnant (table 2). One of the animals gave birth in December 2008. Hormone analysis around this parturition has been done previously and showed significant difference between the hormone levels before and after parturition [Goot, A.C. van der 2009]. For an overview of these hormone profiles, see appendix C.

Mohlaki gave birth around 04-03-2009 and her mean hormone levels dropped from an average of 1273 ng/g DW (SD ±243) during pregnancy to 41 ng/g DW (SD ±31) after parturition. This difference between pregnant and non-pregnant hormone levels seemed to be very significant (two sample *t-test*, unequal variances n= 4, n=5, df= 8 $P < 0{,}001$).

Tharo gave birth around 28-03-2009 and her mean hormone levels dropped from 943 ng/g DW (SD ±269) to 37 ng/g DW (SD ±14). It is very difficult to state anything about the oestrus cycle of Tharo because of a big time gap in between a few samples and because of a probable false sample. Though the difference between her progestagen levels before and after parturition seemed to be very significant (two sample *t-test*, unequal variances n= 4, n=5, df = 7 $P < 0{,}001$). (Table 2)

The progestagen levels of Tharo during pregnancy were 25 times that of the hormone levels during the non-pregnant luteal phase and the progestagen levels of Mohlaki were 30 times higher during pregnancy than during the non-pregnant luteal phase.

Table 2. Mean gestagen concentrations of the six study animals during assumed pregnant (Π) and non-pregnant periods (I)

	I		Π	
Female ID	**Duration-days**	**Mean (± SE) faecal gestagen (ng/g DW)**	**Duration-days**	**Mean (± SE) faecal gestagen (ng/g DW)**
Radimpe	94 21/01/09-24/04/09	25,30 ± 9,05		
Mohlaki	53 06/03/09-28/04/09	40,58 ± 31,09	40 22/01/09-02/03/09	1272,60 ± 243,41
Tharo	20 25/03/09-14/04/09	36,72 ± 13,83	43 26/01/09-09/03/09	942,86 ± 268,99
Munyani	100 22/01/09-01/05/09	68,79 ± 36,07		
Mokibelo	56 05/03/09-01/05/09	40,12 ± 19,02		
Grikie	92 28/01/09-30/04/09	17,05 ± 5,42		

Discussion

The objective of this study was to determine the reproductive status of six free-ranging female white rhinoceroses and to indicate pregnancy using fecal progestagen analyses to gain more information on this subject. Unfortunately this four months study and the regularity of sample collection did not produce sufficient data to give clear information about the length and type of cycles present in free-ranging wild rhinoceroses. This study included six female white rhinoceroses, to gain more significant data the number of animals used in this study could be increased. Therefore female white rhinoceroses should be chosen that are easy to track. I encountered a few white female white rhinoceroses, other than my study animals, frequently during my study. They (such as Pedi and Moruleng) could be added to the study. The fact that there were no regular samples of all the individuals made the determination of the cycles even more difficult. Samples twice weekly are minimally required to determine the oestrus cycles of the animals. The use of telemetry could solve this problem. With the use of telemetry the animals can be found more easily, it would make the search for the rhinoceroses less time-consuming and it would reduce the costs (time and diesel consumption) of the research [Goot. A.C. van der 2009]. Two of the animals had radio transmitters implanted in their horn, but for unknown reasons these transmitters stopped working. It would be beneficial for the ongoing research to investigate what went wrong and to use new tracking systems in all the study-animals. Another way to gain more data could be to decline the number of animals and/or increase the number of researchers and vehicles so that the search for each rhinoceroses could be intensified and samples can be collected more frequently.

There are different cycle lengths recorded for female white rhinoceroses. Patton et al [1999] found two different types. Type I with a cycle length of approximately 35 days and type II with a duration of approximately 65 days, progestagen concentrations during luteal phase (390.0 ± 14.4 ng/g feces) being three times as high as during the interluteal phase (103.9 ±7.8 ng/g feces). The difference between de cycles is presumably attributable to variation in luteal phase length. The type I cycle is presumed to represent the typical oestrus cycle for the white rhinoceroses. Schwarzenberger et al [1998] described four different cycle categories. Type 1 estrus cycles of 10 weeks with high luteal activity (> 800 ng/g feces); type 2 cycles of 4-10 weeks (250-750 ng/g feces); type 3 with no estrus cycle regularity (100-200 ng/g feces), but some luteal activity and type 4 with no luteal activity (< 100 ng/g feces). Brown et al [2001] reported 21-day cycles in a white female rhinoceros. Radcliffe et al [1997] reported an increase in progestagen concentration 7-9 days post ovulation, progestagen concentrations remained elevated for 19-22 days before returning to baseline values, progestagen concentrations remained high after conception and did not decrease until parturition. Ovulation occurred during lowest concentrations of fecal progestin. Brown et al [2001] reported long (70 days) and short (33 days) oestrus cycles in female white rhinoceroses. The follicular phase was variable and lasted from 2-21 days. The average baseline fecal progestagen concentrations found were 1.22 ±0.41 µg/g feces with peak luteal phase concentrations ranging from 3-24 µg/g feces.

Goot A.C. van der [2009] and Charbon Y.N. [2009] drew conclusions from the data they collected from 21-10-2008 until 21-01-2009 from five rhinoceroses also included in this study. Charbon Y.N. [2009] reported that it seemed that Tharo was pregnant, comparing the measured high levels of progestagen with Radimpe who was certainly pregnant. Tharo has been categorized as a type 2 animal as classified by Schwarzenberger et al [1998]. Grikie seemed not to be pregnant and doubt was raised on whether she was cycling or in a lactation anoestrus. Munyani also seemed not to be pregnant although her hormone levels were slightly higher than those of Grikie. They were both categorized as type 4 animals as classified by Schwarzenberger et al [1998]. Mohlaki and Radimpe were both pregnant, concluded from pregnancy tests of their blood samples. Progestagen levels did therefore not indicate luteal phases. Radimpe`s progestagen concentration levels declined to follicular phase values nine to twelve days post partum. [Goot A.C. van der 2009] (See appendix C for hormone profiles)

Despite the difficulty of determining the oestrus cycles, I identified one possible cycle-length of 37 days (Munyani). This approximately corresponds to the type I cycle (35 days) found by Patton et al [1999] and Radcliffe et al [1997] who determined a cycle of 33 days in white rhinoceroses. To determine the oestrus cycles I made the assumption that ovulation occurred in the nadirs of the profile followed by a rise for 7-9 days in progestagens (the assumed luteal phase) and thereafter a decrease in progestagens (assumed follicular phase) [Radcliffe R.W. et al 1997]. The profiles of the non-pregnant females (except Mokibelo) showed nadirs six to eight days preceding the peak levels,

presumably indicating ovulation. During the following six to nine days the progesterone values increased. Thereafter the hormone levels decreased slightly. This shows some resemblance to the findings of Radcliffe et al [1997].

Grikies profile showed very low levels of progestagen. Her hormone levels have been low ever since the start of this long-term study in October 2008, not exceeding the 25 ng/g DW. This seems to indicate that she is in an anestrus. Charbon Y.N. [2009] states that this could be due to a lactation anestrus. After parturition, ovulation and estrus are delayed by the suckling stimulus of the calve which releases oxytocine from the pituitary [Senger P.L. 2003]. Black rhinoceroses show lactation anoestrus for the first three to five months post partum after which cyclicity is resumed, while other perrisodactyla like the horse and the tapir recycle shortly after parturition [Brown J.L. et al 2001]. There is not much information available on the possible duration of the presumable anestrus period after parturition of the white rhinoceros. Since Grikie was already 512 days post partum at the start of this study it seems to be implausable that the anestrus is caused by lactation. Calves suckle approximately until the age of one year [Owen Smith R.N. 1988, Brown J.l. et al 2001]. Suckling by Grikies calve is not seen during this study. If she is experiencing an anestrus period it would presumably be caused by something else.

The peak concentrations of fecal hormones were highly variable in between the different animals, but also within the same animal. The presumable cycle lengths were also variable within and between the animals. Differences in cycle length could be explained by reproductive abnormalities such as a pyometra, endometritis or early embryonic loss which would lead to an extended luteal phase and an extended progestagen secretion. Short luteal phases are associated with low progestagen production caused by anovulation, cystic or hemorrhagic follicles [Brown J.L. et al 2001, Radcliffe R.W. 1997, Patton M.L.1999, Garnier J.N. 2002]. Extended follicular phases are associated with heat stress [Garnier J.N. 2002]. Some free ranging white rhinoceroses do not exhibit estrus behavior when dry conditions prevailed [Owen Smith R.N. 1988]. It was difficult to see if the study animals in this study suffered from these abnormalities, due to the short time period during the data was collected and the fact that no ultrasound observations were possible to confirm or defute such possibilities. Grikies profile showed two peak levels, although not exceeding the 25 ng/g DW. If it could be that she is cycling, she could have had a prolonged cycle, possibly an extended follicular phase. This could be due to heat stress. Although Patton M.L. et al [1999] states that the type II cycles have extended luteal phases, this seemed not to be the case with Grikie because the hormone concentrations did not exceed the mean hormone concentrations after the presumed luteal phase. The abnormalities mentioned above need to be taken into account when analyzing data over a longer period of time. Also stress and social behavior may influence the oestrus cycle, both principally problems in captive white rhinoceroses [Carlstead K. et al 2005]. Females need to be exposed to more than one male to initiate the oestrus cycle. This could be due to the potential lag in the gene pool [Owen Smith R.N. 1988].

Mokibelo`s profile did not show a nadir (presumed ovulation) which indicates that she may not be cycling yet although white rhinoceroses should start to get follicular active at an age of three to four years [Hermes R. et al 2006, Owen Smith R.N. 1988]. We can not conclude for certain that she is not cycling yet, because of the short time period (57 days) that her fecal samples were collected. It could be that she is cycling, but accidentally no nadir was found during the study period.

Two of the studied rhinoceroses were pregnant and gave birth during my study period. This corresponds to the findings of Charbon Y.N. [2009] of Tharo who seemed to be pregnant during her study period and of Goot van der A.C. [2009] who determined pregnancy in Mohlaki. Their hormone levels differed significantly before and after parturition. Goot van der A.C. [2009] also found a significant difference between the hormone levels before and after parturition in Radimpe. The hormone levels during pregnancy in Tharo and Mohlaki seemed to be much higher (25-30 times) than during the non-pregnant luteal phase. Patton et al [1999] measured an increase of 100 times the progesterone levels during pregnancy compared to the non-pregnant luteal phase in female white rhinoceroses. Schwarzenberger et al [1998] also found a considerably higher level of progestagens during pregnancy than during the non-pregnant luteal phase in female white rhinoceroses. Differences between these results could be due to the different procedures of hormone analysis or different periods of measurement. The day of conception for both pregnant rhinoceroses was unclear and thus the gestation lengths are unknown. Schwarzenberger et al [1998] found gestation lengths in white rhinoceroses of approximately 15-16 months and found fecal progestagen levels to be considerably higher as of the fourth and fifth month post conception than during non-pregnant luteal phase. Patton et al ([1999] determined a significant increase in pregnane levels three months post conception. Thus fecal progestagen measurement can be used as a diagnostic tool for pregnancy detection. I suggest that an amount of approximately three fecal samples during 1 week are collected of a probable pregnant female to measure the progestagen levels. If the progestagen levels exceed the non-pregnant luteal phase concentrations by 30 times, it can be assumed that the specific white rhinoceros is pregnant.

The hormone levels of Mohlaki two days after parturition were 1,14% of the levels two days before parturition. This is almost similar to a finding of Patton et al [1999] in which the pregnane values of a pregnant female dropped one day after parturition to 2,4 % of the values observed at the end of pregnancy

A period of lactational anestrus after parturition is the likely explaination for the low values seen in the hormone profiles of Tharo and Mohlaki after parturition. Radimpe`s hormone profile showed only one hormone peak level, this could also be due to the fact that she calved in November and could still have been in a lactation anoestrus. The death of a calf shortly after birth shortens the period of reduced reproductive activity, as was the case with Munyani [Garnier J.N. et al 2002].

The hormone profiles (especially Tharo`s profile) of the pregnant rhinoceroses showed a time gap in sampling after parturition. White rhinoceroses retreat in the thicket after parturition to protect their offspring and are therefore more difficult to find [Owen Smith R.N. 1988]. Tharo`s hormone levels were inconsistent with the estimated date of parturition which was nineteen days after the drop in fecal hormone levels. The lag time in rhinoceroses between the actual increase in blood hormone levels and the fecal measurements of this increase, due to the enterohepatic circulation and the fecal steroid excretion, is 24 to over 48 hours [Schwarzenberger F. et al 1996]. Radcliffe et al [1997] reports the gastrointestinal transit time as 65 hrs for white rhinoceroses and Patton et al [1999] also reports a lag time of 48 to 72 hours. Even if we take the lag time in account then still it can not explain the very different information on Tharo on the day of parturition. This could mean that either the samples on day 48 was a 'false' sample (it was either from a different rhinoceroses than Tharo or collected when they were older than an hour or there were faults in storing or processing the samples) or we overlooked the calve and therefore the day of parturition could be before day 48. The sample collected on day 24 seemed to be either of a different rhino (sample was collected on tracks, defecation was not recorded) or could be a fault in storing or processing the sample. Although 100 percent certainty about each sample is strived for, by following tracks of the animal and collecting dung from its track it is not guaranteed that the sample is of the right animal. Tracks sometimes mix up between different individuals in the same area and make following the right animal more difficult.

Birth intervals of white rhinoceroses are reported to be 2.6 years. The calf accompanies the cow until shortly before the next parturition, then the cow chases the calf away before she gives birth [Owen Smith R.N. 1988]. I experienced this with Mohlaki, who chased her calf away before she gave birth to a new calf in March. Tharo remained with her older calf after she gave birth to a new calf in March. This situation was very extraordinary but made tracking of and identifying the animal easier. If a cow did not produce a calf over a four year period it could be presumed that she either is infertile or had aborted during pregnancy or lost her calf shortly after birth.[Owen Smith R.N. 1988] The latter is the case for Munyani who after loosing her second calf shortly after birth, accepted her first calf (birth date 04-11-2003) to join her again. She seemed to be cycling again and should be conceiving in the near future

This study is still ongoing today in the Lapalala Wilderness game reserve and more reproductive data of the six female white rhinoceroses will be collected. Another interesting aspect to study at a later stage could be the possible determination of the gender of the fetus by fecal steroid analysis. This would be of great interest to wildlife management, for anticipating possible translocations. Duer C. et al [2007] and Meyer J.M. et al [2004] indicated differences in the maternal serum progesterone concentrations in Asian elephants based on fetal gender. The differences in serum progesterone concentrations between cows carrying male and female calves was significant during the first and second phase of gestation (20-65 weeks). Elephants have a gestation period of 20-23 months [Hermes R. et al 2007]. If I compare this to white rhinoceroses with a

gestation length of approximately 16 months this would mean that differences in progesterone concentrations could be measured during the fourth-eleventh month. The difficulty may be the notification of conception which would be necessary to determine the phase of gestation. The determination of the gender of the calve after parturition is done by sighting and this information is not always rapidly available. Of the two cows that gave birth during my study it was still unknown what the gender of their calves was.

Prolonged periods of high glucocorticoid concentrations in response to chronic stressors is supposed to be a cause of low reproductive rates in female white rhinoceroses in captivity. The possible influence of stress on fecal progesterone concentrations could be interesting to measure in free-ranging white rhinoceroses. Fecal glucocorticoid measurement has been validated for this species [Turner J.W. et al 2002, Carlstead K. et al 2005].

Conclusion

Determining the oestrus cycle of the free-ranging female white rhinoceroses in this study was problematic. Two of the six study animals were pregnant and were therefore not cycling. The fact that it was to difficult to get regular samples (ideally two or three per week) together with the short time period of collecting samples made determining the oestrus cycles about impossible. Thereby the data that do exist on the oestrus cycle of female white rhinoceroses are conflicting and therefore difficult to compare with. The use of telemetry could enhance tracking and finding the animals and alleviate the problem of finding the animals and collecting regular samples.

Although the number of animals in this study is limited, the results indicate that fecal progestagen measurement can be used to indicate pregnancy in free-ranging white rhinoceroses. This will be of great value for wildlife management and thus conservation of the species. Further research is necessary to see if the gender of the calf can also be determined.

Since my study is part of a long-term study in reproduction of the female white rhinoceroses in Lapalala Wilderness, more regular samples could be obtained and therefore the outcome of the overall study might increase the basic knowledge of female reproductive patterns of female wild rhinoceroses whilst free-ranging.

The oestrus cycle can be influenced by a number of factors such as reproductive abnormalities and (heat)stress. If there are conflicting cycles found at the end of this research it could be due to stress as is found in captive animals. To investigate the probable effect of stress on free-ranging white female rhinoceroses further research needs to be done.

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Appendix A

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. <u>Antibody coating on microtitre plate (MTP):</u>
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. <u>Reagents (stock solutions – Standards, Second Antibody & Biotin label):</u>

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 1.13.) into each well. The blue colour turns yellow.

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

Appendix B

RESEARCH ON WHITE RHINO (*Ceratotherium simum simum*) IN LAPALALA

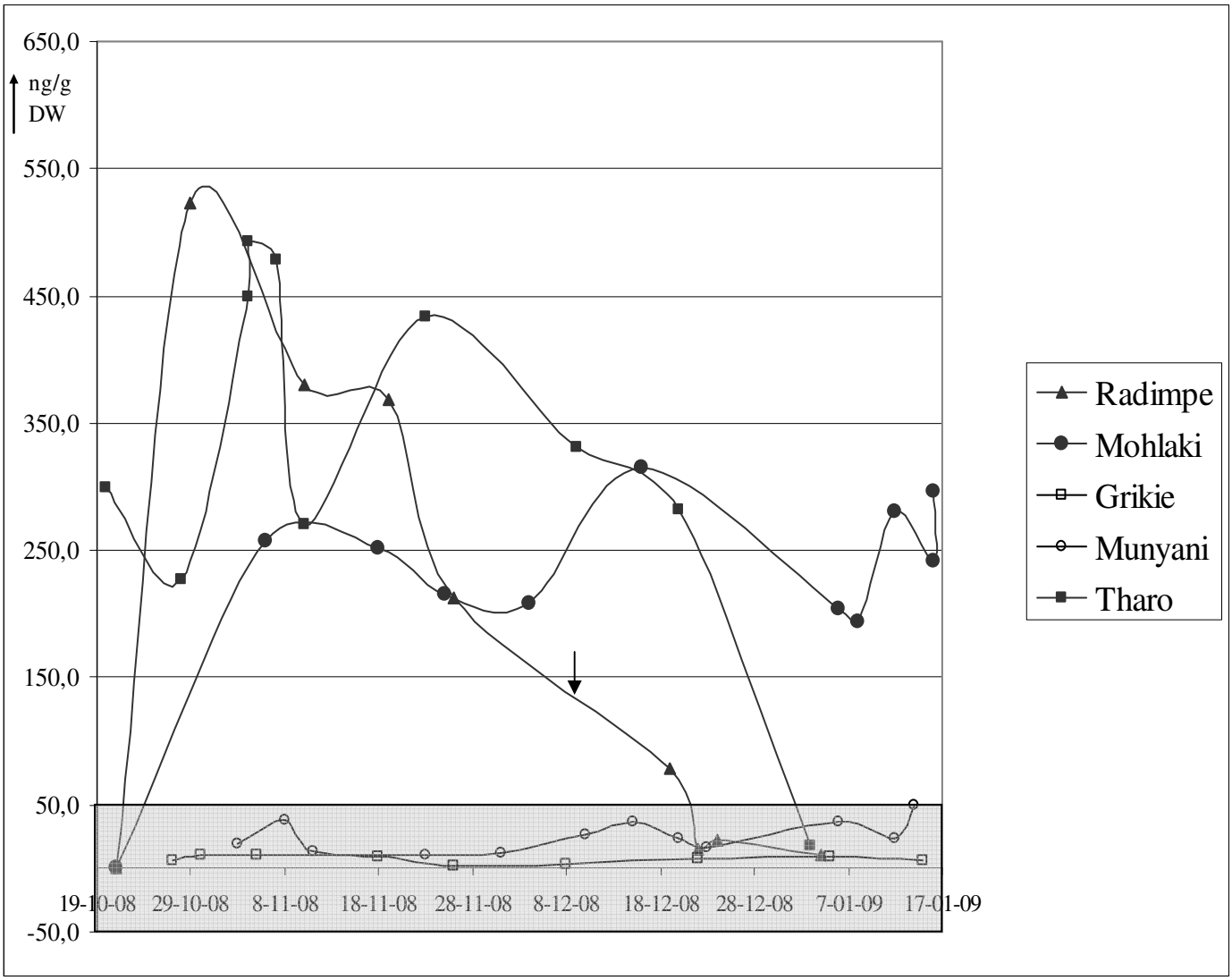
List of Faecal Samples, weights, and Immunoreactive Hormone Concentrations in Rhino Faeces (ng per g dry weight)

Name of Individual	Sample No.	Date	Reproductive Status	Weight (mg)	Gestagen concentration (ng/g DW)
Radimpe	R 1	21-1-2009	Post Partum	55	**29,1**
Radimpe	R 2	27-1-2009	Post Partum	52	**18,6**
Radimpe	R 3	29-1-2009	Post Partum	57	**33,4**
Radimpe	R 4	4-2-2009	Post Partum	56	**16,6**
Radimpe	R 5	17-2-2009	Post Partum	54	**30,6**
Radimpe	R 6	2-3-2009	Post Partum	52	**15,1**
Radimpe	R 7	10-3-2009	Post Partum	58	**26,2**
Radimpe	R 8	17-3-2009	Post Partum	56	**27,4**
Radimpe	R 9	25-3-2009	Post Partum	53	**33,7**
Radimpe	R 10	31-3-2009	Post Partum	59	**29,7**
Radimpe	R 11	9-4-2009	Post Partum	54	**10,7**
Radimpe	R 12	15-4-2009	Post Partum	53	**41,4**
Radimpe	R 13	24-4-2009	Post Partum	59	**16,3**
Mohlaki	Ma 1	22-1-2009	Pregnant	51	**1215,3**
Mohlaki	Ma 2	26-1-2009	Pregnant	52	**911,0**
Mohlaki	Ma 3	12-2-2009	Pregnant	52	**1553,7**
Mohlaki	Ma 4	18-2-2009	Pregnant	54	**1258,3**
Mohlaki	Ma 5	2-3-2009	Pregnant	59	**1424,7**
Mohlaki	Ma 6	6-3-2009	Post Partum	59	**16,2**
Mohlaki	Ma 7	23-3-2009	Post Partum	52	**85,8**
Mohlaki	Ma 8	1-4-2009	Post Partum	58	**34,9**
Mohlaki	Ma 9	28-4-2009	Post Partum	59	**25,4**
Tharo	T 1	26-1-2009	Pregnant	58	**665,2**
Tharo	T 2	3-2-2009	Pregnant	50	**961,2**
Tharo	T 3	13-2-2009	Pregnant	50	**39,9**
Tharo	T 4	26-2-2009	Pregnant	54	**1202,2**
Tharo	T 5	9-3-2009	Pregnant	52	**56,0**
Tharo	T 6	25-3-2009	Pregnant	57	**22,4**
Tharo	T 7	2-4-2009	Pregnant	50	**46,0**
Tharo	T 8	8-4-2009	Post Partum	58	**29,9**
Tharo	T 9	14-4-2009	Post Partum	51	**29,3**
Grikie	G 1	28-1-2009	Unknown	58	**22,4**
Grikie	G 2	10-2-2009	Unknown	57	**7,2**
Grikie	G 3	19-2-2009	Unknown	50	**24,1**
Grikie	G 4	3-3-2009	Unknown	57	**17,2**
Grikie	G 5	16-3-2009	Unknown	52	**12,6**
Grikie	G 6	24-3-2009	Unknown	60	**18,7**
Grikie	G 7	2-4-2009	Unknown	56	**14,2**

Name of Individual	Sample No.	Date	Reproductive Status	Weight (mg)	Gestagen concentration (ng/g DW)
Grikie	G 8	9-4-2009	Unknown	55	**18,7**
Grikie	G 9	15-4-2009	Unknown	52	**12,4**
Grikie	G 10	30-4-2009	Unknown	60	**22,9**
Munyani	Mu 1	22-1-2009	Unknown	54	**92,8**
Munyani	Mu 2	26-1-2009	Unknown	51	**86,4**
Munyani	Mu 3	6-2-2009	Unknown	54	**59,6**
Munyani	Mu 4	9-2-2009	Unknown	51	**39,3**
Munyani	Mu 5	19-2-2009	Unknown	51	**89,5**
Munyani	Mu 6	23-2-2009	Unknown	52	**166,7**
Munyani	Mu 7	5-3-2009	Unknown	55	**42,3**
Munyani	Mu 8	12-3-2009	Unknown	58	**54,0**
Munyani	Mu 9	16-3-2009	Unknown	52	**63,8**
Munyani	Mu 10	23-3-2009	Unknown	53	**27,5**
Munyani	Mu 11	1-4-2009	Unknown	59	**104,9**
Munyani	Mu 12	7-4-2009	Unknown	57	**79,3**
Munyani	Mu 13	14-4-2009	Unknown	59	**40,3**
Munyani	Mu 14	21-4-2009	Unknown	63	**35,0**
Munyani	Mu 15	1-5-2009	Unknown	59	**50,4**
Mokibelo	Mo 1	5-3-2009	Unknown	58	**69,5**
Mokibelo	Mo 2	16-3-2009	Unknown	54	**32,6**
Mokibelo	Mo 3	23-3-2009	Unknown	56	**63,4**
Mokibelo	Mo 4	1-4-2009	Unknown	55	**20,0**
Mokibelo	Mo 5	7-4-2009	Unknown	55	**15,6**
Mokibelo	Mo 6	14-4-2009	Unknown	50	**35,3**
Mokibelo	Mo 7	21-4-2009	Unknown	55	**46,5**
Mokibelo	Mo 8	1-5-2009	Unknown	54	**38,0**

Appendix C

Hormone profiles of five female white rhinoceroses collected from October 2008-January 2009



Arrow indicates date of parturition of Radimpe: 10-12-2008