Non-invasive monitoring of reproductive function in four free-ranging female white rhinoceroses *(Ceratotherium simum simum)* by analyzing faecal progestagen metabolite levels

Part of a long term study by IBREAM

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The reproductive rate of captive white rhinoceroses is suboptimal and to address this issue it is essential to gain insight in the reproductive physiology of the rhinoceros. Therefore in this study the objective was to non-invasively try to determine the cycle length of free-ranging female rhinoceroses. Four female free-ranging rhinoceroses in South Africa were monitored by analyzing faecal progestagen metabolites. This study was performed during a period of three months and is part of a long-term study by IBREAM (Institute for Breeding Rare and Endangered African Mammals; www.ibream.org). In order to obtain a correct hormone profile, faecal samples needed to be collected at a frequency of 2-3 times a week. In earlier stages of this long-term study six rhinoceroses were monitored. However the sample frequency was insufficient, subsequently the number of animals was narrowed down to four. This study shows that minimizing the study animals to four individuals leads to an adequate outcome to perform this research, in which the sample frequency is sufficient. The faecal progestagen metabolites were measured with an Enzyme Immunoassay (EIA), providing a progestagen profile of the four rhinoceroses (i.e. Grikie, Munyani, Mokibelo and Radimpe). With the method used in this study the cycle length of one rhinoceros (Grikie, approximately seven weeks) could be determined, thereby implying that the other three are not cycling. In the case of Munyani mating behaviour was observed, raising the impression that she in fact was cycling at that time. Anoestrous ís actually plausible for the remaining two rhinoceroses Mokibelo and Radimpe in view of their reproductive background history. Regarding these two rhinoceroses the results of this study correspond with our expectations contrary to Munyani. Obviously more than one cycle is required in order to determine a norm for the cycle length of the white rhinoceros. Thus prolongation of the research is essential.

Keywords: Faecal progestagen metabolites, Ceratotherium simum, non-invasive monitoring, oestrous cylce, white rhinoceros

Introduction

As of December 2007, the white rhino population constituted about 17,475 animals and is currently listed as Near Threatened on the IUNC Red List of Threatened Species [Milliken et al., 2009; IUNC Red List, 2010]. According to this estimation 16,273 animals out of the 17,475 occur in South Africa (93%). In 2009 a provisional estimate is done for South Africa indicating 18,553 white rhinoceroses [Milliken et al., 2009].

The present worldwide rhinoceros population consists of five species; the white rhinoceros (*Ceratotherium simum & Ceratotherium cottoni)* (Fig. 1), the black rhinoceros (*Diceros bicornis, Diceros michaeli & Diceros minor*), the greater one-horned rhinoceros (*Rhinoceros unicornis)*, the lesser one-horned rhinoceros (*Rhinoceros sondaicus & Rhinoceros annsmiticus*) and the Sumatran rhinoceros (*Dicerorhinus sumatrensis & Dicerorhinus harrissoni*) [Milliken et al., 2009].

Figure 1. Southern white rhinoceros *(Ceratotherium simum simum)* in Lapalala.

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Although there appears to be some success in conservation efforts (IUNC Red List, 2010; Milliken et al., 2009], three of the five extant species are listed Critically Endangered, namely the black rhino, the lesser one-horned and the Sumatran rhino [IUNC Red List, 2010]. The listing on the IUCN list is due to several threats to the rhinoceros population, such as poaching (Fig. 2), hunting, habitat degradation and fragmentation, as well as civil wars [Hermes et al., 2009; Milliken et al., 2009; IUNC Red List, 2010; Brown et al., 2001; Garnier et al., 2002; Schwarzenberger et al., 1998].

The white rhinoceros population continued to increase in the wild with a average annual net growth rate of 7,2% since 1995 until 2009 except for Zimbabwe, where the numbers are declining [Milliken et al., 2009]. However this population growth is in danger due to the current poaching situation. The number of illegally killed white rhinoceroses in 2010 is the highest ever experienced in South Africa. 323 white rhinoceroses have been lost poaching in 2010, this is nearly three times the number of rhinoceroses killed in 2009 in South Africa [WWF, 2011]. The cause of this alarming situation lies in the heightened demand of rhino horn in Asia. Recently unsubstantiated reports are coming in that the horn of a rhinoceros has cancer-curing qualities [WWF, 2011].

Declining state budgets for conservation and declining capacity in some areas could also seriously threaten the numbers. Therefore one should not be complacent about their conservation. [Milliken et al., 2009; IUNC Red List, 2010]. This study aims to help conserve the species on the long term, as will be further illustrated below.

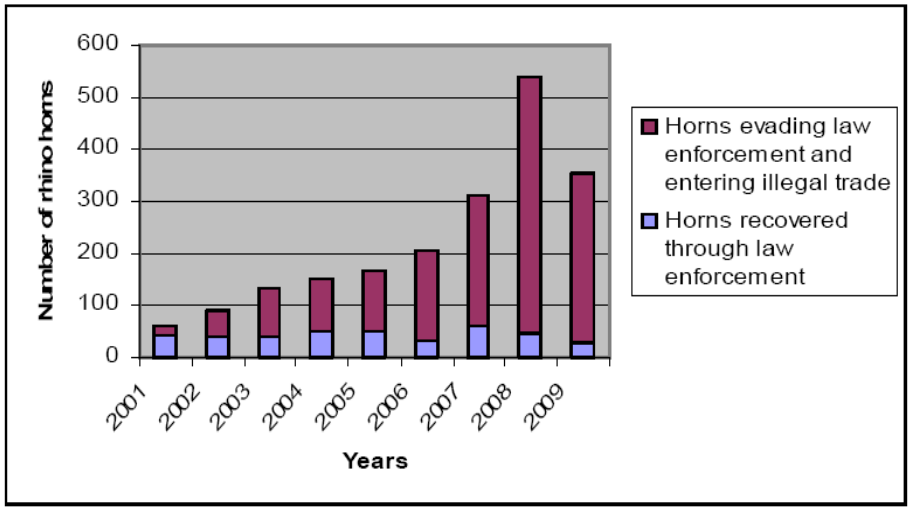


Figure 2. Estimated minimum number of rhino horns recovered and lost to illegal trade in Africa, 2001-2009 [Milliken et al., 2009]

In contrast to the wild, the captive population has a negative annual growth rate of 3,5% and is in a demographic crisis [Hermes et al., 2009; Schwarzenberger et al., 1998]. Rhinoceroses in captivity have a low reproductive success, especially among those born in captivity and the reasons for this subfertility and reproductive failure are unclear [Hermes et al., 2009].

To elucidate the causes of the subfertility, reproductive evaluation of both male and female white rhinoceroses has become of high priority in recent years [Portas et al., 2005] Continuous efforts are made to understand the reproductive anatomy and biology and the accompanying pathologies [Portas et al., 2005; Hermes et al., 2009].

It is important to establish viable ex situ populations, as they may be needed as a

reservoir for reintroduction into the wild, and research may prove invaluable for the management of the wild as well as the captive population.
The information that is available on the reproductive physiology of captive white rhinoceroses is limited and the available data are contradictory, especially regarding the length of the reproductive cycle [Schwarzenberger et al., 1998; Patton et al., 1999]. A study conducted by Radcliffe and co-workers (1997), in which they combined ultrasonographic examinations and faecal progestagen metabolite analysis on one white rhinoceros, showed two nonconceptive cycle lengths of 31 and 35 days. Whereas another study on 16 white rhinoceroses suggests a 10-week cycle length using faecal progestagen metabolite analysis [Schwarzenberger et al., 1998]. In contrast, another study based on faecal progestagen metabolite analysis using 13 white rhinoceroses, argues that 1 month is the typical cycle length [Patton et al., 1999]. Finally the study of Brown and co-workers (2001), on 13 white rhinoceroses using faecal steroid metabolite analysis, indicates both short (1 month) and long (10 weeks) cycles.

One explanation for the paucity of these data is that majority of the females are pregnant for the greater part of their reproductively active period and thereby rarely exhibit non-conceptive cycles, whereas the remaining animals are often non-reproductive and their cycles are erratic or absent (in anoestrous) [Patton et al., 1999]. Various studies found that approximately half of the white rhinoceros females fail to show any evidence of ovarian cyclicity, [Brown et al., 2001; Hermes et al., 2007; Schwarzenberger et al., 1998; Patton et al., 1999].

As a consequence of this, it can be challenging to find data representative of normal reproductive patterns. The main objective of our study was to address this lack of data by conducting a long-term study in which we non-invasively try to determine the length of the oestrous cycle of four free-ranging white rhinoceroses during their intergestation period by analyzing faecal progestagen metabolites. Due to the poor breeding performances of the white rhinoceros held in captivity, it is preferable to study the rhinoceros in their natural environment when it comes to characterizing the reproductive cycle. When knowing the cycle length of a free-ranging white rhinoceroses, one gets an indication of what to expect for the cycle length of a captive rhinoceroses. More information on the cycle length of the captive white rhinoceros can help establishing viable ex situ populations which can be reintroduced into the wild when wild populations are sincerely threatened.

The study described here is spread out over three months (August 2009 until November 2009) and is part of a long term study of IBREAM (Institute for Breeding Rare and Endangered African Mammals; www.ibream.org), Utrecht University and the University of Pretoria, which started in October 2008.
The preceding researchers of this study, i.e. A.C. van der Goot, 2009; Y.N. Charbon, 2009; B. Bitter, 2009, and M.M. Meijers, 2010, state that non-invasive faecal progestagen metabolites are a correct indicator of reproductive activity.

Materials and Methods

Study site

The study was conducted at a vast privately-owned nature reserve, called Lapalala Wilderness (Fig. 3), which is situated in the mountains of the Waterberg area (Limpopo Province) 3,5 hours north of Johannesburg (Google Maps: Lapalala Wilderness, Waterberg, Limpopo, South Africa).
Due to the arrangements made by IBREAM with Lapalala Wilderness it was

possible to conduct the research here. These arrangements were provided by the supervisor of this study, Monique Paris, Research Director of IBREAM.
Lapalala Wilderness was founded in 1981 and is now part of the UNESCO Waterberg Biosphere Reserve. This approximately 36,000 hectare game reserve is home to white (approximately 40) and black (approximately 20) rhinoceroses and today is one of the country's leading private rhino sanctuaries [Lapalala Wilderness, Lapalala.com].

Figure 3. Lapalala Wilderness in summer.

Due to financial constraints and the gazetting of land claims over a large proportion of the reserve, Lapalala is no longer open for tourism. In consequence, the animals are exposed to little of human interaction since 2005.
The climate in Lapalala Wilderness is subtropical, and the annual wet season extends from November to March, with a mean annual rainfall estimated at 500mm [Lapalala Wilderness, Lapalala.com].

Study animals

The study started with the monitoring of six supposedly reproductively active female rhinoceroses (October 2008 – August 2009), but from August 2009 the number of animals was narrowed down to four. The reason for this was the low sample frequency obtained when studying six animals, which as a consequence produced insufficient data [Bitter, 2009; Meijers, 2010]. The four rhinoceroses that were selected for this study were the ones that were most easily traced and identified in order to increase the sample frequency to an optimum. A paired *t*-test is used to compare the weekly sample frequency before and after reducing the study animals. The downside of reducing the number of animals is that it becomes more questionable if it can be translated to the general female white rhinoceros.

All four female rhinoceroses are thought to be sexually mature with ages varying from six to twenty years and all but the youngest, Mokibelo, reproduced in the past. No lactating was seen in the study animals during this study period, which leads to suspect that the calves are no longer suckling. Therefore lactation anoestrous, i.e. oestrous and ovulation are delayed by the suckling stimulus of the calf the first 3-5 months of lactation, is presumably not applicable for these females [Brown et al., 2001].
Specific details on the individual life history are given in Table 1.

Grikie is the oldest rhino in the study and she already produced 5 calves. White rhinoceros can remain fertile well into their twenties [Wilson et al., 2010], Grikie being twenty (Table 1) should still have a couple of years to reproduce. Since her youngest calf is two years old at the beginning of this study period (August 2009) it is likely for her to become pregnant shortly. The gestation period for rhinoceroses is approximately 16 months [Hermes et al., 2007; Patton et al., 1999; Wilson et al., 2010]. When you add up the two years and the gestation period you approach Grikie's mean interval between births, hence it is plausible for her to be fertile or even to have fallen pregnant again.
Munyani is a hand-reared rhino and she produced two calves in the past. Alas, her youngest calf died shortly after birth which made her first calf Mokibelo return to her mother again. The date of parturition of Munyani's youngest calf is unknown, but is estimated to be three years ago. The

interval between the first and second calf is estimated to be two years and nine months, therefore Munyani might get pregnant in the near future if she is not already carrying.

Mokibelo, Munyani's first calf, is six years old and has not yet produced a calf. In the wild, a female white rhinoceros will attain sexual maturity around six years of age [Wilson et al., 2010]; consequently it is questionable whether she is already cycling or pregnant.

Radimpe is a fourteen year old rhino that produces three calves in de past. With her youngest calf being only eight months at the beginning of this study it is improbable for Radimpe to get pregnant at this moment. Taking her previous interbirth intervals into account odds are it will take another year for her to get impregnated.

Radimpe was included in the long term study when she was pregnant (Oktober 2008) and has been a part of this study ever since. It is interesting to determine how long post-partum she will stay in anoestrous, when luteal activity will start and when she will become pregnant again.

Table 1. Background information on the animals used in this study.

Rhino	Date of birth	No. of calves	Date of birth calves	Mean interbirth interval	Days post partum
Grikie	01-01-1989	5	1: 01-01-1993 2: 01-01-1997 3: 01-06-1999 4: 01-10-2002 5:18-08-2007 (=youngest calf)	± 3 years and 5 months (±1250 days)	724
Munyani	01-02-1996	2	1: 04-11-2003 2:Estimated July 2006 (†) (=youngest calf)	± 2 years and 9 months (±1000 days)	± 1125
Mokibelo	04-11-2003	n/a	n/a	n/a	n/a
Radimpe	01-04-1993	3	1: 07-02-2003 2: 25-02-2006 3: 10-12-2008 (=youngest calf)	± 2 years and 11 months (±1066 days)	257

Data collection

The aim was to get as many samples from every rhino each week and preferably not on consecutive days. The results from two samples on consecutive days are very likely to be similar, whereas the results from samples taken one or two days apart might show a pattern. To obtain a correct hormone profile a sample frequency of 2-3 samples a week is necessary [Bitter, 2009; Meijers, 2010]. Together with an experienced guide the rhinoceroses were tracked twice every day, starting at 5:20 am and 1:20 pm. Every rhino has a home range and most of the time the rhino can be found in this area. The home range of a female white rhinoceros is approximately 20 km^2 and the core area is 5 km^2 [White et al., 2007]. So in order to find the animal we drove to this core area and started looking for tracks. Our guide was able to identify the rhinoceroses by their characteristic footprint (Fig. 4). If tracks were found or the animal was spotted we proceeded on foot. Unfortunately this was not always the case; it was not uncommon to be driving for hours without any trace of the specific rhino, particularly on rainy days (Fig. 10, where the occasions search-found are plotted against each other). To positively identify the rhino when spotted, external features were used, e.g. earnotches, horn, general shape of the

body, size of the calf accompanying her and on occasion behavioural characteristics of the rhinoceros could be used for identifying. After a few weeks of following these four rhinoceroses, recognition by the students will also take place after one glance.

Figure 4. Footprint rhinoceros (below) and human (European size 40) (above).

After the identity was confirmed a quick backtrack took place, hereby looking for fresh faecal samples. The sample was exclusively collected within one hour of defecation, when it was still warm and no insect contamination had occurred. Since defecation was not seen, these samples are less reliable and fortunately a mere handful of these samples were taken.
In all other cases the rhinoceros was followed until defecation was seen (Fig. 5), meanwhile trying not to disturb the animal. In order to minimize the disturbance it is best to stay downwind and keep the noise down.

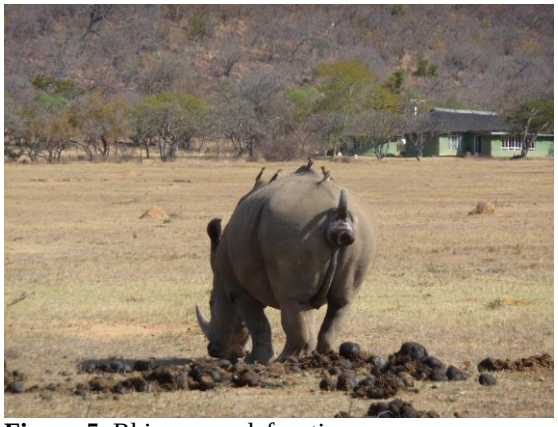


Figure 5. Rhinoceros defecating.

As soon as the rhino moved a minimum of fifteen meters away from the faeces it was safe to collect (Fig. 6). Although studies revealed there is no significant difference in faecal progestagen metabolite concentration between the central portion and the outer layer of the faeces ball [Schwarzenberger et al., 1998], the inner part is preferred to minimize the possible effect of insect contamination on the outside. Ten to forty grams faeces were collected using gloves (Hartmann Peha-soft, REF:942150), whilst removing as much undigested material as possible. The samples were stored in a glass container and immediately placed in a cooler box. On the return to base camp (an average of 1hour and 18 minutes after defecation) the animal, time and date were noted on the container and the samples were stored frozen at -20°C until analysis.

Figure 6. Collecting a fresh sample with glove.

Faecal extraction

When the field work was finished, after nearly three months of collecting in Lapalala Wilderness, the samples were taken to the Laboratory situated in Pretoria (University of Pretoria, Faculty of Veterinary Science situated on the Onderstepoort campus, South Africa) to be further processed and made suitable for the Enzyme Immunoassay (EIA) and the endocrine assessment (Appendix A).
The first step is to lyophilize the samples for 48-96 hours in a vacuum oven (Instruvac Freeze-drier from Air & Vacuum Technologies, Pretoria, SA, Model: VFDT 02.50) at -54°C with vacuum set at approximately 672 Torr

(Fig. 7). By freeze-drying the samples you remove the moisture content from the faecal samples in order to reduce the variability in weight due to the liquid in the samples. Following lyophilisation, the dry matter was pulverized by hand and sieved through a nylon mesh to separate the fine faecal powder from any existing fibrous material (Fig. 7).

Figure 7. Vacuum oven (left) and sieving the dry samples (right).

To avoid cross-contamination during the pulverization the surface and the utensils, i.e. tweezers, sieve and gloves were cleaned with 80% EtOH (prepared from Ethanol Absolute 99%, Merck, Saarchem, diluted with distilled water) between each sample.
Approximately 0.05g (0,05-0.058g) was weighed of each sample and transferred to a sample tube (Kimble Borosilicate Glass, Disposable Culture Tubes, 12x75mm). The precise weight of each sample was entered and used to calculate the exact faecal progestagen metabolite levels per gram dry weight (Fig. 8).

Figure 8. Weighing 0,05g of each sample.

Following in the extraction 3 ml of freshly prepared 80% ethanol in distilled water was added to each sample tube. The mixture of faecal powder and 80% EtOH was then placed in a shaker and vortexed on high speed for fifteen minutes. Thereafter the tubes were placed in a centrifuge for ten minutes on 3000 RPM.
To complete the extraction 1.5 to 2 ml of the supernatant was transferred to an Eppendorf tube utilizing a pipette. In anticipation for the measurement of immunoreactive faecal progestagen metabolites via microtiterplate enzyme immunoassay, the Eppendorf tubes were stored upright at -20°C.

Hormonal analysis

The faecal progestagen metabolite analysis was performed using a double antibody technique described by Prakash et al. (1987), unfortunately this method has yet to be validated for the species rhinoceros. Faecal extracts were analysed using an indirect Enzyme Immunoassay (EIA) [Szdzuy et al., 2006]. Briefly, the EIA was performed using a polyclonal antibody (rabbit) that was raised against 5α-pregnan-3β-ol-20-one-3-HS-BSA. The microtitre plate (MTP) was coated with this primary antibody. The antigen (Ag) will bind to this antibody. The labelled secondary antibody was a 5α-pregnan-3β-ol-20-one-3-HS-peroxidase label, which is comparable to a biotin label. The binding of this secondary antibody to the Ag generates an enzyme reaction.
Assay sensitivity, based on 90% of maximum binding, was 4pg per well and the intra- and inter-assay coefficient of variation for the assay ranged between 9.33% and 16.46%.
The immunoreactive faecal progestagen metabolite concentrations are expressed as ng per g dry weight (DW) of rhinoceros faeces (Appendix B).

First data analysis method
(accepted method)

The mean concentrations ± the standard deviation (SD) of faecal progestagen metabolites were calculated for all four

rhinoceroses for the complete period of the long term study (oktober 2008 until November 2009). Definition of the oestrous cycle was based on faecal progestagen metabolite profiles, meanwhile bearing in mind the background of the specific rhino.
For each study animal, a nonpregnant baseline faecal progestagen metabolite value was calculated using an iterative process in which values greater than the mean plus 2 standard deviations (SD) were removed [Brown et al., 1999; Moreira et al., 2001; Powell and Monfort, 2001; Brown et al., 2004; De Haas van Dorsser et al., 2007; Meijers, 2010]. The average should be subsequently recalculated and the elimination process repeated until no value exceeds the mean plus 2 SD. The mean of the remaining values is the baseline faecal progestagen metabolite concentration [Meijers, 2010].
The first point after values increased above the baseline faecal progestagen metabolite concentration and remained elevated for at least 2 consecutive weeks marked the onset of the luteal phase (LP) [Brown et al., 2001; De Haas van Dorsser et al., 2007]. The commencement of the follicular phase (FP) was defined as the first of two consecutive faecal progestagen metabolite concentrations falling below baseline concentrations [Brown et al., 2001].
Oestrous cycle length was calculated as the beginning of one follicular phase to the beginning of the next. Since the sampling did not occur every day, it is not feasible to determine the length without a slight deviation of two to four days.
On occasion, subjective observations were used to distinguish between an actual luteal phase and random fluctuations in the data. Behavioural observations are also included in characterization of the cycle, for instance observations of mating behaviour, e.g. copulation; mounting, can be suggestive for the onset of the luteal phase [Patton et al., 1999].

Second data analysis method (experimental method)

In this study a second data analysis method is tested. The data are analysed in the same manner as the method described above with one adjustment, i.e. instead of the faecal progestagen metabolite having to be elevated for two consecutive weeks, it has to be elevated for merely one week. This method is an experiment and is tested for only this study period (August 2009 until November 2009).

Results

Data collection

In de beginning of this long-term study it proved to be difficult to collect a sufficient amount of samples from the six free-ranging animals. To increase the sample frequency we reduced the number of study animals to four (Fig. 9). When the sample frequency per week increases (Table 2), the hormone profile can be calculated more accurately. A paired *t*-test comparing the weekly sample frequencies before and after reducing the study animals results in a p-value of 0,001. The null hypothesis stating no difference in sample frequencies is rejected at $p<0,05$. Therefore the weekly sample frequency after reducing the study animals is significantly higher then before reducing the study animals.
In addition the sample frequency was subject to the functioning of the vehicle. In order to search and track an animal the vehicle needed to be reliable, which was not always the case. Whenever the vehicle was not functioning no samples could be collected, whereas when it did function the efficiency of the study is fairly high as can be seen in figure 10. For the rest the efficiency of the research was left to the tender mercy of the rhinoceros (Fig. 10).

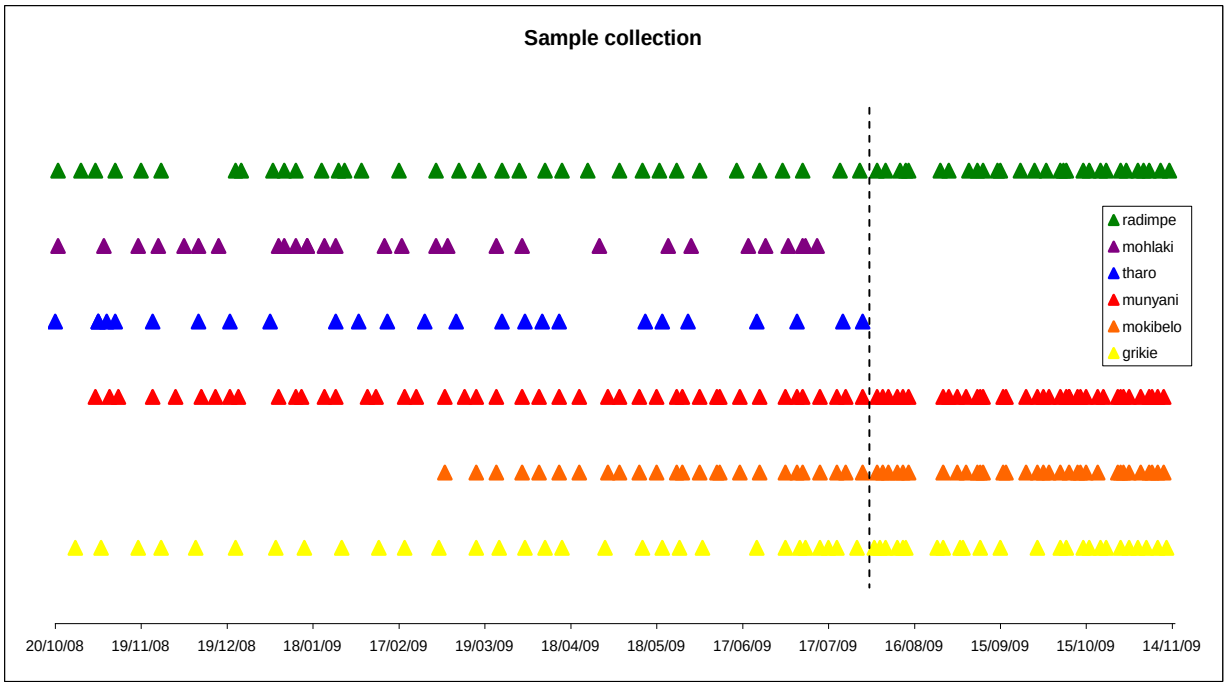


Figure 9. Sample collection during the entire study period. Every triangle represents the collection of a sample. The dashed line indicates the moment of study animal reduction.

Table 2. Sample frequency before and after reduction of the study animals

	Tharo	Moklaki	Radimpe	Grikie	Munyani	Mokibelo
Sample frequency per week Oct '08 - Jul '09	0,625	0,725	0,875	0,75	1,125	1,238
Sample frequency per week Aug '09 - Nov '09	n/a	n/a	1,813	1,563	2,313	2,2

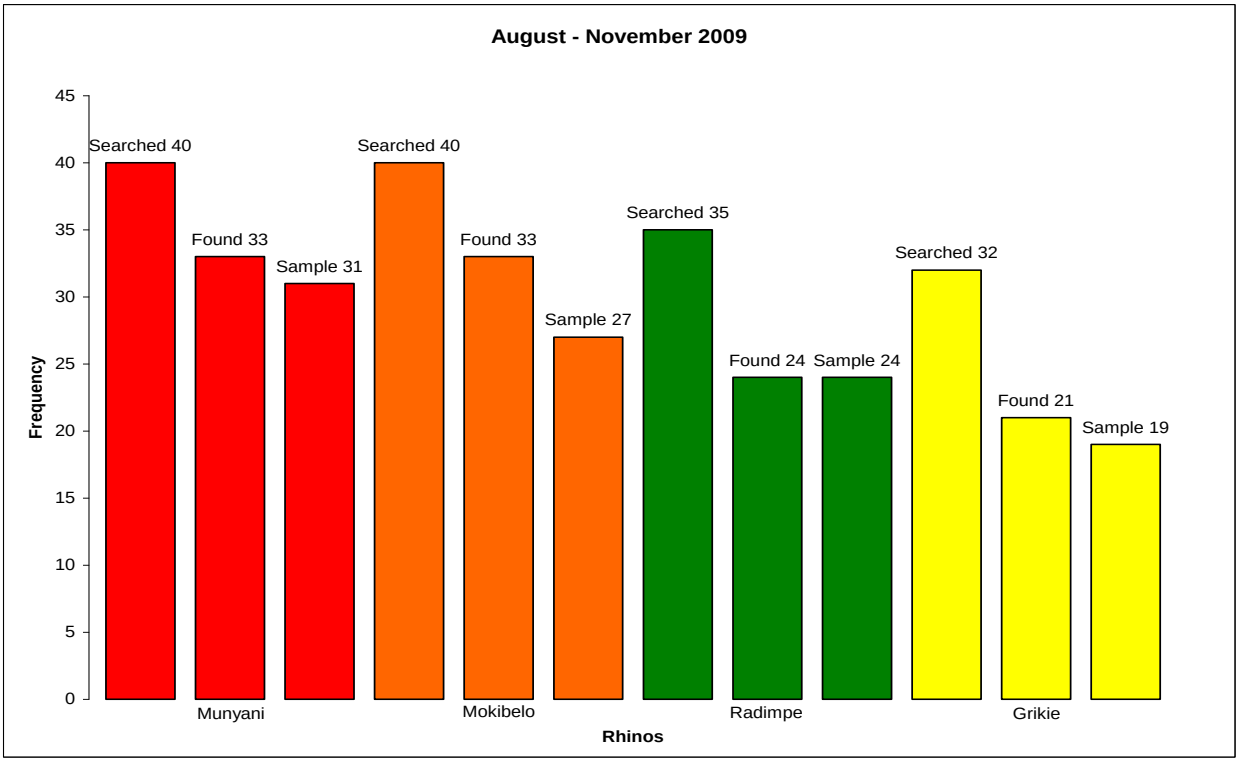


Figure 10. Research efficiency in the period August 2009 – November 2009. Munyani: 31/40= 77,5%; Mokibelo: 27/40= 67,5%; Radimpe: 24/35= 68,6%; Grikie: 19/32= 59,4%.

Individual hormone profiles

After calculating a baseline faecal progestagen metabolite concentration for all four rhinoceroses the data were analysed using the abovementioned two methods.

The baseline faecal progestagen metabolite concentration calculated for Grikie was 560,99 ng/g DW (Fig. 13). The cycle length, measured with the first method, from the end of one luteal phase until the following, respectively 26-8-2009 until 16-10-2009, is 51 days (± 7 weeks). Given that sampling did not occur daily, the actual cycle length might deviate with a few days. The second method gives the same result.
Several times Grikie was seen together with a dominant bull, showing behaviour suited to a cycling cow. Frequent urinating by Grikie was observed and the bull was permitted to come close and sniff up and lean against Grikie's behind. No real mating was observed, although at the last taken sample (12-11-2009) the bull came very close to mounting (Fig. 12).

Munyani's baseline faecal progestagen metabolite concentration was set at 2975,83 ng/g DW (Fig .14). No luteal phase could be determined for Munyani with the first method. The highest peak only remained above baseline for 9 days. Munyani was accompanied by a dominate bull on several occasions and mounting and actual copulation was observed on 26-10-2009. A couple of days postmating the faecal progestagen metabolite level started to rise. Unfortunately the last sample was taken on 11-11-2009 and at this point the faecal progestagen metabolite had been elevated for merely 8 days.
When the second method is applied two luteal phases can be detected and a cycle length can be determined. The first luteal phase starts on 6-10-2009 and the commencement of the second is 3-11-2009. Taken the indisputable deviation into

consideration the cycle length is speculated to be approximately 4 weeks.

The baseline faecal progestagen metabolite concentration measured for Mokibelo was 2166,91 ng/g DW (Fig. 15). In this animal there was also no period of elevated faecal progestagen metabolite for two consecutive weeks, thus no cycle length can be determined for Mokibelo when using the first analysis method. One high peak returned to baseline concentration after 11 days.
With the experimental method we can find one luteal phase for mokibelo starting 17-9-2009 and ending 6-10-2009. The previous luteal phase ends 20-7-2009, hence this cycle length is roughly 11 weeks long. Given that Mokibelo was always together with Munyani, she automatically came in contact with Munyani's bulls. But according to our observations the bulls were always focused on Munyani and never showed interest in her calf.

A baseline faecal progestagen metabolite concentration of 1935,24 ng/g DW was calculated for Radimpe (Fig. 16) Since the partus of her youngest calf Radimpe's faecal progestagen metabolite level remained low. Faecal progestagen metabolite kept fluctuating around baseline concentrations and no luteal phase could be determined with the first analysis method. However when we apply the second analysis method, two luteal phases are discovered. These phases start respectively on 7-10-2009 and 6-11-2009, hence the cycle length is roughly 4 weeks.
On occasion Radimpe was seen together with other rhinoceroses, usually a group of rhinoceroses and never alone with one male and no mating behaviour was observed.

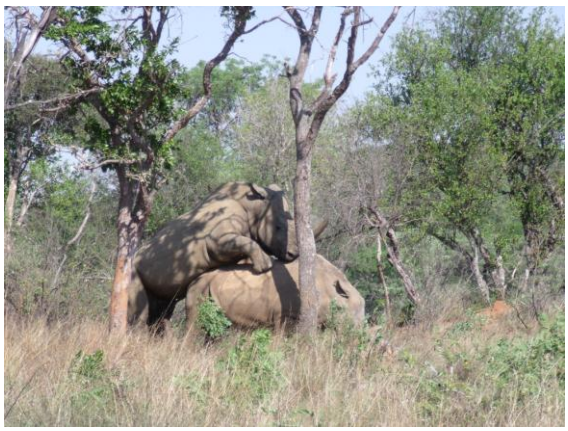


Figure 11. Poacher and Munyani mating (26-10-2009)

Figure 12. Grikie (left) and Poacher (right) showing mating behaviour (12-11-2009)

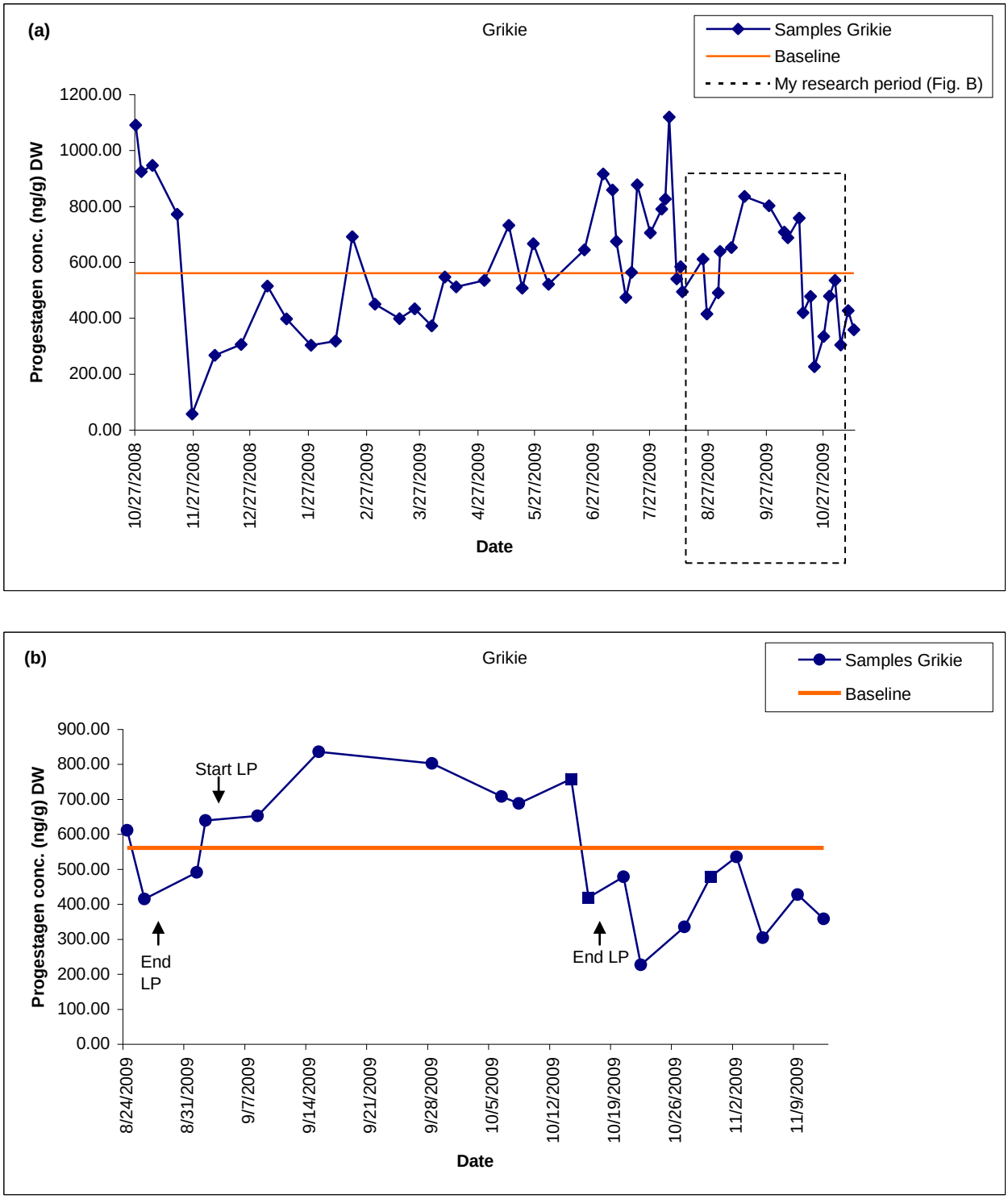
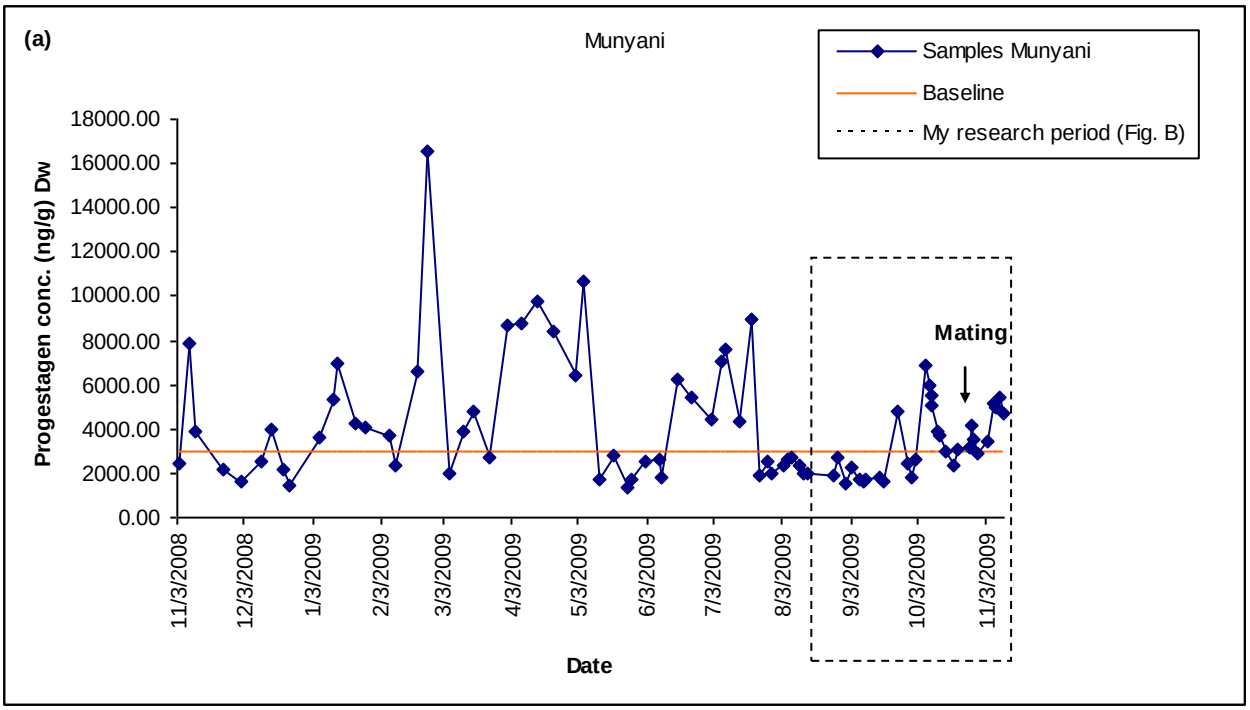


Figure 13. Grikie - Faecal progestagen metabolite profile during the whole study period (Oktober 2008 – November 2009) (**b**), and faecal progestagen metabolite profile my research period: August – November (**a**) (The samples indicated by a square instead of a circle are samples collected on spore and therefore less reliable (14-10-2009; 16-10-2009; 30-10-2009))



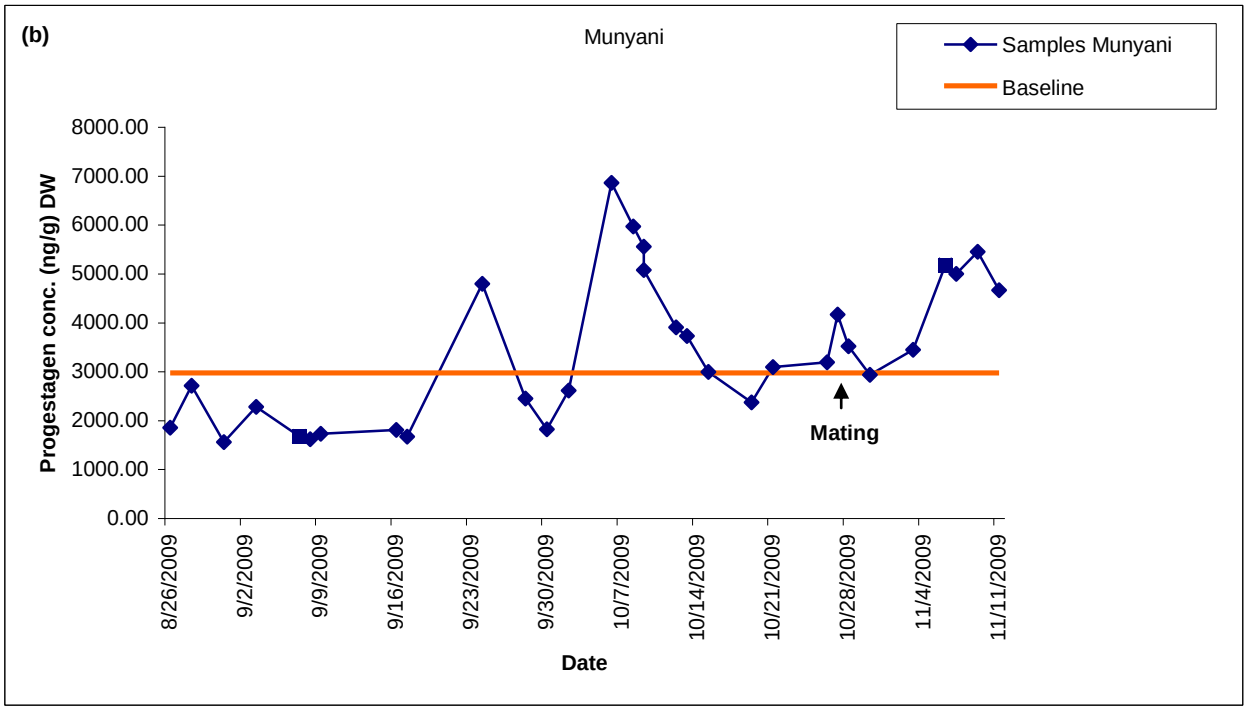
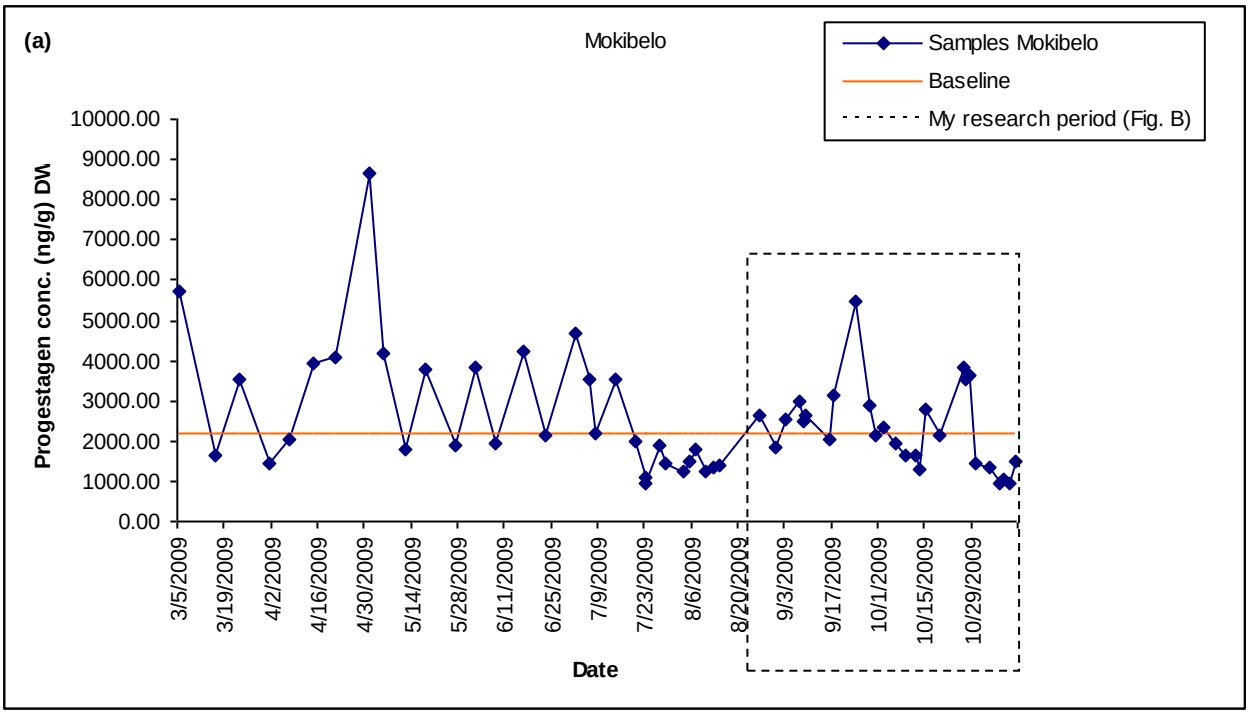


Figure 14. Munyani - Faecal progestagen metabolite profile during the whole study period (Oktober 2008 – November 2009) (**a**), and faecal progestagen metabolite profile during my research period: August – November (**b**) (The samples indicated by a square instead of a circle are samples collected on spore and therefore less reliable (7-9-2009; 6-11-2009))



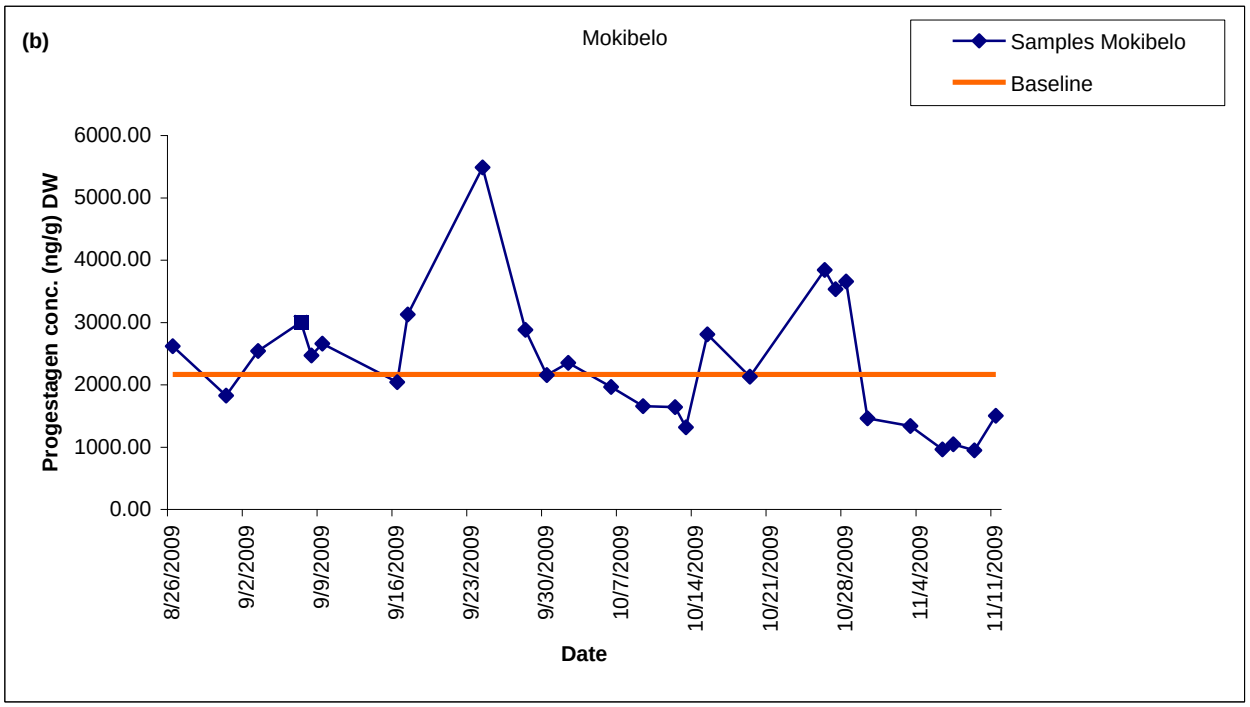
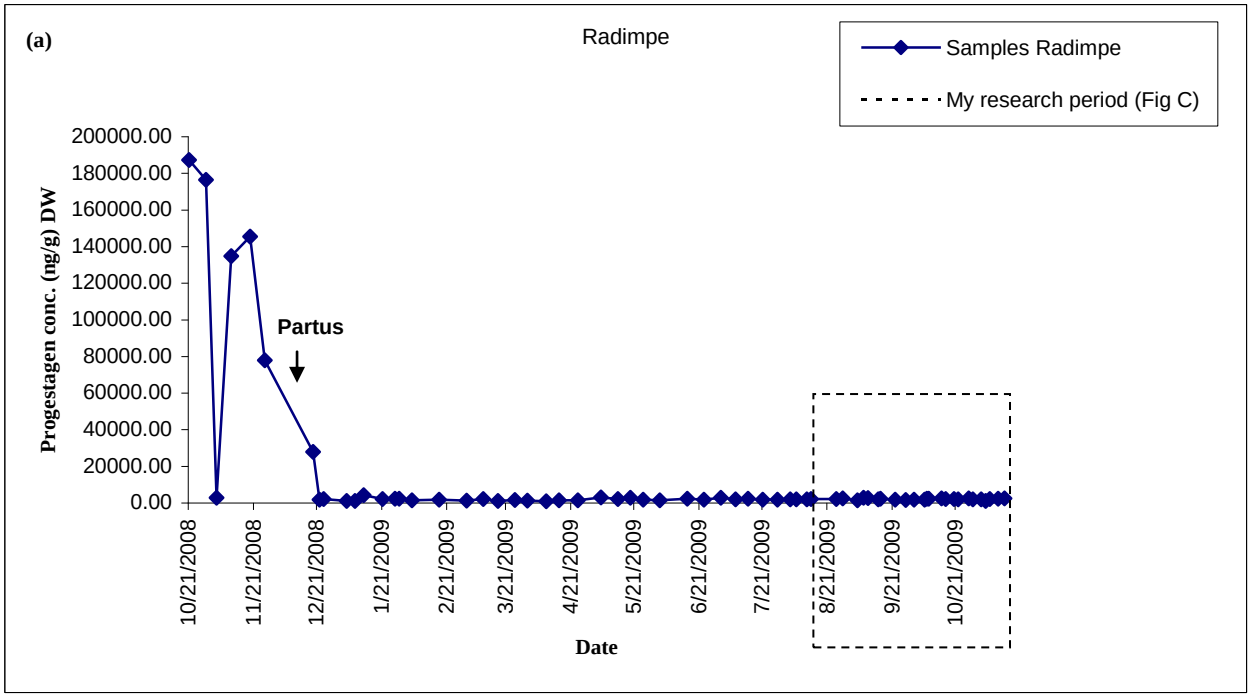
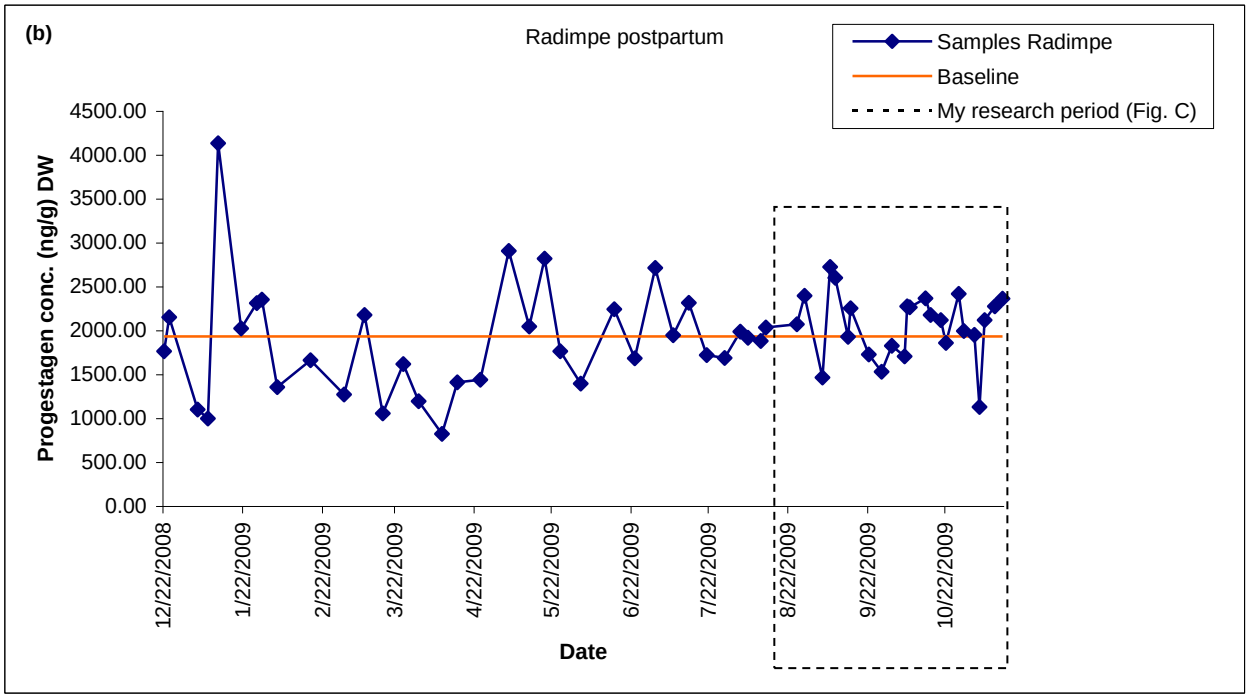


Figure 15. Mokibelo - Faecal progestagen metabolite profile during the whole study period (May 2009 – November 2009) (**a**), and the faecal progestagen metabolite profile during my research period: August – November (**b**) (The sample indicated by a square instead of a circle is a sample collected on spore and therefore less reliable (7-9-2009)).


(a)
Radimpe
Samples Radimpe
My research period (Fig C)
Progestagen conc. (ng/g) DW
200000.00
180000.00
160000.00
140000.00
120000.00
100000.00
80000.00
60000.00
40000.00
20000.00
0.00
Partus
10/21/2008
11/21/2008
12/21/2008
1/21/2009
2/21/2009
3/21/2009
4/21/2009
5/21/2009
6/21/2009
7/21/2009
8/21/2009
9/21/2009
10/21/2009
Date


(b)
Radimpe postpartum
Samples Radimpe
Baseline
My research period (Fig. C)
Progestagen conc. (ng/g) DW
4500.00
4000.00
3500.00
3000.00
2500.00
2000.00
1500.00
1000.00
500.00
0.00
12/22/2008
1/22/2009
2/22/2009
3/22/2009
4/22/2009
5/22/2009
6/22/2009
7/22/2009
8/22/2009
9/22/2009
10/22/2009
Date

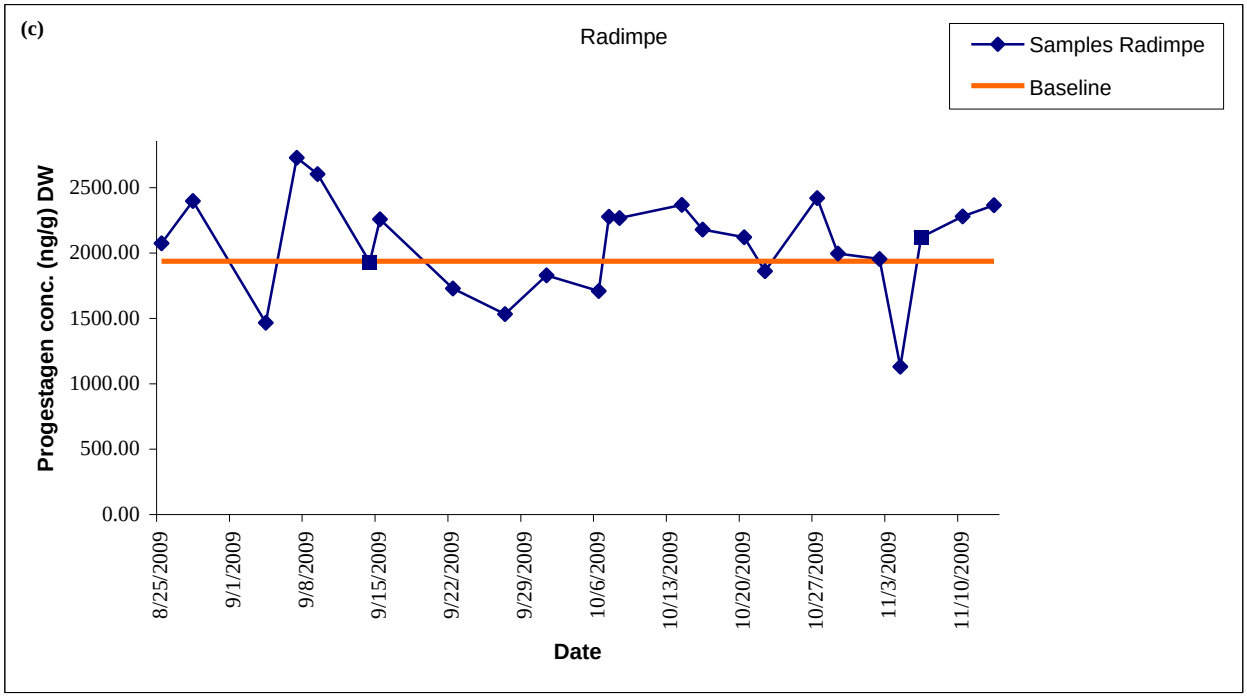


Figure 16. Radimpe - Faecal progestagen metabolite profile during the whole study period (Oktober 2008 – November 2009) (**a**), the faecal progestagen metabolite progestagen profile postpartum (10-12-2008 – November 2009) (**b**), and the faecal progestagen metabolite profile during my research period: August – November (**c**) (The samples indicated by a square instead of a circle are samples collected on spore and therefore less reliable (14-09-2009; 6-11-2009)).

Discussion

Data collection

The markedly increased sample frequency, as given in Table 2 after the reduction of the study animals, demonstrates that it was the right decision to decrease the number of study animals in order to collect sufficient samples. By reducing the number of study animals from six to four, the number of samples collected per week were approximately doubled. The paired *t*-test proves that the weekly sample frequency is significantly higher after reducing the study animals. In earlier stages of this long term study the sample frequency was found to be an obstacle, however the sample frequency we achieved is adequate to perform this study.

As can be seen in figure 10 the efficiency of this research is fairly high, however the research suffered from the unreliability of the vehicle. With a reliable car it is possible to collect more samples, hereby improving the research significantly.

As stated above in earlier stages of this long term study the sample frequency lies too low to gain sufficient data [Bitter, 2009; Meijers, 2010], therefore there is only a period of a few months with adequate sample frequency. Hence it would be advisable to extent the research conducted in manner of the final period.

Evidently a higher number of study animals would provide more significant data which can be implemented to the general white rhinoceros species. This can be achieved by conducting the research in the same manner as done in the last period of this long term study, however more people and extra vehicles are a prerequisite. My prediction is that the data can be duplicated with the help of one extra researcher, one guide and one vehicle or two quads.

Individual hormone profiles

One cycle length of about 7 weeks was determined in the period August until November for Grikie. The second analysis method gives the same result. To establish an average cycle length for Grikie more cycles must be detected and to realise this, the research must be prolonged.

Grikie's overall faecal progestagen metabolite level lies significantly lower than the other three rhinoceroses and it lacks high peaks. One could think Grikie is a "flatliner", a term given to describe the observation of stable, baseline concentrations of serum progestagen indicative of ovarian inactivity [Brown, 2000]. However this is inconsistent with Grikie's reproductive background. The fact that Grikie produced several calves in the past and exhibited mating behaviour is highly suggestive of ovarian activity. Furthermore the determination of a cycle in this research invalidates the theory of Grikie being a "flatliner".

When Brown (2000) uses the term "flatliner" it refers to elephants, and this does not automatically apply to rhinoceroses.

With the first method used to analyse the data no cycle could be determined for Munyani. Taken her reproductive background into account and the fact that mating has been observed during the study period, it is very probable that in fact she is cycling. Perhaps this method is too meticulous for interpreting these data. With the second experimental analysis a cycle length of approximately

4 weeks is determined. The cycle length of 4 weeks corresponds with the results of several previous studies, e.g. Radcliffe et al. (1997), Patton et al. (1999) and Brown et al. (2001) whom also found monthly cycles. As previously mentioned one cycle is not sufficient to establish the cycle length with certainty, hence the research should be extended. Evidently this second analysis method is merely experimental and the calculated cycle length is only a speculation. Perhaps in the future the methods can be tested further to create a optimal method for analysing the data.

The youngest rhino in the study, Mokibelo, seems according to the data not properly cycling as yet. The faecal progestagen metabolite profile during the whole period of this research is fairly irregular. With the first method used no true cycle could be determined in the period August until November. With the second method we can find a cycle length of roughly eleven weeks long. Taken her background into account it is questionable if Mokibelo is in fact cycling. Also the fact that she is still together with her mother is an indication she might not be entirely sexually mature. Another theory is that since Munyani has not reproduced a new calf she has not renounced Mokibelo and this being together with her mother decelerates Mokibelo's maturation.
When considering the above-mentioned, the existence of the cycle determined with the second analysis method becomes questionable. Perhaps this experimental method is not accurate.

As expected Radimpe's faecal progestagen metabolite profile postpartum shows no high peaks and fluctuates around the baseline. Given the fact that Radimpe has a young calve and did not show mating behaviour, it is suggested that it is plausible she was still in anoestrous. The fact that no luteal phase was discovered with the first analysis method and therefore no cycle length could be determined validates this prediction.
However when we apply the second analysis method a cycle length of roughly 4 weeks is determined. Since, based on the reproductive background data, there is every indication that Radimpe is in fact not cycling, the accuracy of this experimental analysis method becomes questionable.
Radimpe's mean interbirth interval is nearly 3 years, therefore she is expected to get pregnant again around summer 2010, however luteal activity might already be commencing. As aforementioned a cycle length of approximately 4 weeks has been suggested for Munyani and has been determined in previous studies (i.e. Radcliffe et al. (1997), Patton et al. (1999) and Brown et al.(2001)). To validate this theory the study on Radimpe must be prolonged, preferably until she gets pregnant again.

Conclusion

Of all four rhinoceroses one cycle length was determined in the period August – November 2009 with the accepted analysis method. With the experimental analysis method cycle lengths for all four rhinoceroses can be determined. However as stated in the discussion, the actual existence of these cycles is questionable. Thus this experimental analysis method is too uncertain to draw any conclusions from and the accepted analysis method remains operative. Obviously if we like to learn more about

the normal reproductive patterns of the white rhinoceros more then one cycle length must be determined and additional data is required. In order to gain this additional data the data collection period should be prolonged. There are reasons to think that one other rhinoceros, Munyani, is also cycling. To discover her cycle length the study needs to be prolonged and perhaps the study method has to be adjusted, e.g. usage of another faecal metabolite. If the estimations about Mokibelo and Radimpe are correct, they will become more interesting in the near future from a reproductive point of view.

The way the research was conducted during this study period (August – November 2009) has proven to be efficient. With one car a sufficient number of samples can be collected from four rhinoceroses, therefore the advice is to continue in this way.

If the number of study animals is to be increased in order to gain more significant data which can be implemented to the general white rhinoceros species, more researchers and more cars are required.

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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.