DETERMINING THE REPRODUCTIVE STATUS OF TWO FEMALE SOUTHERN WHITE RHINOCEROSES (*CERATOTHERIUM SIMUM SIMUM*) IN KHAO KHEOW OPEN ZOO IN THAILAND BY MEASURING FECAL PROGESTERONE

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Breeding performance in captive southern white rhinoceroses is disappointing. The same applies to the two female southern white rhinoceroses in the Khao Kheow Open Zoo in Thailand. Since these females have never produced offspring, the reproductive status is examined in this study. With the use of non-invasive monitoring, by measuring fecal progesterone and by observing behavior related to the estrous, one could make some assumptions about their reproductive cyclicity.
The peaks and nadirs in the progesterone profiles, indicate that both females are cyclic, with cycle lengths of respectively 30,5 days and 36 days. Furthermore, both females showed mating behavior, which occurred on the day progesterone concentrations were at a nadir (taking in account the gastrointestinal transit time), underlining that they were in estrous. However, the timespan of this study and the regularity of sample collection were not sufficient to draw firm conclusions. A long-term study with a more regular sample collection would give more comprehensive hormone profiles. Simultaneously performing ultrasonographic examination would provide valuable data to gain more knowledge about the failure in reproducing of the two female southern white rhinoceroses.

Keywords: white rhinoceroses, *Ceratotherium simum simum*, reproduction, progesterone

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Introduction

The white rhinoceros is one of the five rhinoceros species that exist. The species included in the Rhinocerotidae are the African white rhinoceros *(Ceratotherium simum)* which is divided into the two subspecies the Southern white rhino *(Ceratotherium simum simum)* and the Northern white rhino *(Ceratotherium simum cottoni)*, the black rhinoceros *(Diceros bicornis)*, the Sumatran rhinoceros *(Dicerorhinus sumatrensis)*, the Javan rhinoceros *(Rhinoceros sondaicus)* and the Indian rhinoceros, also known as the greater one-horned rhinoceros *(Rhinoceros unicornis)*. All of the five species are documented on the IUCN Red List of Threatened Species, whereas the Indian rhinoceros has the status 'vulnerable' with an increasing population, the Sumatran, black and Javan rhinoceros have the status 'critically endangered', whereas the population of the Sumatran rhinoceros is decreasing, the population of the black rhinoceros is increasing and the trend of the population of the Javan rhinoceros is unknown. The white rhinoceros is listed as 'near threatened' with an increasing population trend. The greatest threat to the rhinoceros population is poaching for their horns, which are used in traditional Chinese medicine and ornamental use [Emslie and Brooks 1999; IUCN].

Although the white rhinoceros is the least endangered with latest estimated numbers that have increased to 20.150 up from 17.500 in 2007 , it was hunted near extinction by the end of the 19th century and there is still concern about the increasing poaching threat [Emslie 2011]. To maintain a viable population of white rhinoceroses reduction of illegal hunting and protection of wild populations is of major importance. Secondly, captive rhinoceroses can serve as a reservoir for reintroduction into the wild, so another major approach in conservation of the white rhino is to enhance reproduction in captivity. Furthermore captive breeding institutions play an important educational role in raising public awareness of the situation of wild rhinoceroses and learning more about this species' biology [Emslie and Brooks 1999].

Unlike their wild counterparts, the reproductive rate of captive rhinoceroses is highly variable, with only few facilities having remarkable success [Patton et al., 1999; Brown et al., 2001]. Annual growth rates in wild populations are 6-10%, whereas growth rates in captive white rhinoceroses is at negative 3.5% [Emslie and Brooks 1999]. These low rates are especially due to the poor breeding performance of captive-born females, as the founding captive population of the white rhinoceros, given appropriate husbandry and management, reproduce better [Schwarzenberger et al., 1998; Patton et al., 1999; Swaisgood et al., 2006]. Furthermore, the higher reproduction rate in founders is also largely due to the import of pregnant animals from the wild [Schwarzenberger et al., 1998].
The reasons for the disappointing captive-breeding are still unclear. Of high importance is understanding the reproductive status of the captive-born population and the factors that influence fertility.

Despite a long history of evaluating fertility of female white rhinoceroses, there is still a lot unclear about the cyclicity and data are contradictory, especially with regards to the estrous cycle.
Schwarzenberger et al. [1998] analyzed fecal progesterone metabolites and with these results they classified rhinoceroses into four categories. The first (1) are the regular estrous cycles of 10 weeks duration and high luteal activity, next (2) estrous cycles between 4-10 weeks and moderate luteal activity, (3) no apparent cycle regularity, with some luteal activity and (4) no apparent luteal activity. Females in category 1 and 2 appeared to ovulate, in which category 2 can conceive, whereas fertility of category 1 remains to be determined. Category 3 and 4 animals did not ovulate, which suggests that anovulation could be a major problem in captive white rhinoceroses as nearly two-thirds of the studied animals fall in this category.
Patton et al. [1999] described two categories by measuring fecal progesterone metabolites and observing behavioral patterns; Type I are cycles of approximately 1 month in duration and Type II with cycles of approximately 2 months. They found that the latter is characterized by extended luteal phases lasting more than twice as long as the Type I luteal phases. Because the Type I cycles are predominant in their results, they suggest that this is the typical reproductive cycle for this species.
Morrow et al. [2008] reported a cycle length between 26 and 38 days, with an average of 31.6 ± 0.6 days, which was characterized using fecal progesterone concentrations and observations of courtship behavior. Also long cycles of (67.2 ± 1.3 days) were detected.
It is generally believed that the 30–35 day cycle can be fertile [Radcliffe et al., 1997; Schwarzenberger et al., 1998; Patton et al., 1999], where on the other hand pregnancy has not yet been documented following a mating associated with a 65–70 day cycle [Schwarzenberger et al., 1998; Brown et al., 2001; Roth, 2006; Morrow et al., 2008].

The difference in two cycle lengths cannot simply be attributed to individual animal differences [Roth, 2006], since some reports describe several females exhibiting both cycle lengths over a period of months. [Schwarzenberger et al., 1998; Patton et al., 1999; Brown et al., 2001; Roth, 2006; Morrow et al., 2008] Patton et al. [1999] suggested that extended luteal phases could be the result of pyometra, endometritis or early embryonic loss (which may be caused by uterine inflammation), all of which have been diagnosed in white rhinoceroses exhibiting prolonged progesterone excretion [Radcliffe et al., 1997; Patton et al., 1999; Brown et al., 2001]. Other factors that may influence the estrous cycle are stress, disturbed behavior, nutrition and ageing [Schwarzenberger et al., 1998; Patton et al., 1999; Hermes et al., 2006]. For example, in females over 20 years of age, the longer cycle length appears to be more prevalent and may be a characteristic of aging animals [Schwarzenberger et al., 1998; Brown et al., 2001] that have long non-reproductive periods [Hermes et al., 2004, 2006; Roth, 2006; van der Goot, 2008]. In these females also the acyclic or 'flat liner' pattern appears to exist more, wherein progesterone remains at baseline concentrations for extended periods ranging from several months to years [Schwarzenberger et al., 1998; Patton et al., 1999; Brown et al., 2001; Roth, 2006; Carter et al., 2007]. However, not all acyclic animals are aged [Brown et al., 2001; Roth 2006], and some information suggests that cyclicity can resume in some females when they are introduced to new males [Patton et al., 1999; Roth 2006].

Several studies used fecal progesterone concentrations to examine reproductive cyclicity, because progesterone increases during the luteal phase of the cycle and is low during estrous [Radcliffe et al., 1997; Schwarzenberger et al., 1998; Patton et al., 1999; Brown et al., 2001; Roth 2006]. Radcliffe et al. [1997] reported that progestin concentrations increased 7–9 days after ovulation without conception and remained elevated for 19-22 days before returning to baseline concentrations. Ovulation occurred at the nadir of the fall of fecal progestin concentrations.
Monitoring progesterone or progestin metabolites has proven effective across all rhino species and has been the most reliable hormone for studying reproductive status of white rhinoceroses [Radcliffe et al., 1997; Schwarzenberger et al., 1998; Patton et al., 1999; Brown et al., 2001; Roth, 2006; Carter et al., 2007]. Combined with behavior observations it has provided valuable information for breeding management [Morrow 2008].

The aim of the current study was to investigate the cyclicity by making a profile of the reproductive status of the two female white rhinoceroses in the Khao Kheow Open Zoo (KK Zoo) in Sri Racha, Thailand, as these females have never reproduced in the KK Zoo before and as the success of captive breeding management becomes increasingly important.
We measured progesterone levels of fecal samples of the females and studied behavior associated with changes in the reproductive cycle, like typical estrous behavior as lifting of the tail to the site, allowance of close approaches by the male and 'standing' behavior with mounting and mating by the male [Radcliffe et al., 1997].

Materials and methods

1. *Study site and animals*

This research was performed in the Khao Kheow Open Zoo (KK Open Zoo) in Ampur Sriracha, Thailand. This zoo is 2000 acres in size and most animals live in large enclosures. The rhinoceros-enclosure is approximately 2000m² with sandy surface, a little grass and two water pools. Here the four southern white rhinoceroses, are housed together day and night. They can be visited by the public from 09.00h until 17.00h (18.00h in weekends) and the visitors can manually feed the rhinoceroses over the 1,5 meter high fence that separates them from the rhinoceroses. The amount of food the public can feed is calculated in advance and consists of corn, grass, beans and bananas. The keepers feed the rhinoceroses daily a calculated amount of horsepellets. The rhinoceroses have unrestricted access to water, because the waterpools are filled up with fresh water every day.

Fig 1. The 1,5 m high fence, where the public can manually feed the rhinoceroses.

The rhinoceroses which are studied are the two females, Som Sri and Kanoon. They have lived together with one male in the KK Open Zoo for almost 15 years. No offspring was produced during that time. The male died of unknown disease in October 2007, after which the females were housed with no male for almost 2 years. Two new males were introduced in 2009.

Table 1. Information on the rhinoceroses in the KK Open Zoo.

Animal	Date of birth	Location of birth	Type of birth	Date of arrival in KK Open zoo	Number of offspring
Som Sri ♀	April 1992	South Africa	Wild born	October 1994	0
Kanoon ♀	March 1993	South Africa	Wild born	September 1994	0
Ingocy ♂	June 2000	Singapore	Captive born	April 2009	Unknown
Zudy ♂	November 2004	Singapore	Captive born	April 2009	Unknown
No name* ♂	March 1993	South Africa	Wild born	September 1994	Unknown

*This animal died of unknown disease in October 2007

To assess the body condition of the rhinoceroses, the standardized body condition scoring system for black rhinoceros, which has been described by Reuter and Horsepool [1996] and has been modified by Reuter and Adcock [1998], is used (Appendix B). Both studied rhinoceroses are scaled in condition score 4, which is a good condition.

2. *Behavioral observation*

The behavior of the rhinoceroses was observed from 04/10/2011 until 07/12/2011 to detect estrus behavior. During the day, from 07.00h until 18.00h the rhinoceros-keepers were at the exhibit and watched the behavioral changes, which are related to the estrous. In addition, I observed the behavior everyday for three hours, from 8.00h until 9.00 and from 16.00h until 18.00h. The estrous signs payed attention to are: approaching of males to females without fighting, chin resting and rubbing from the males on the females back with 'standing' behavior of the females, females showing swollen vulva with vaginal discharge, urine squirting and curling or lifting the tail to the side, mounting from the male on the female and mating itself.
The observed signs were noted and uses for determining the estrous cycle length.

3. *Sample collection*

Fecal samples from both females were collected as often as possible, with an average of 3 times a week. The rhinoceros-keeper took fresh samples of approximately 50 grams after direct observation of defecation, thus confirming the right identity of the sample. Since Schwarzenberger et al. [1998] found that the progesterone metabolite concentration within the feces does not differ significantly between the central portion and the outer layer of the fecal ball, the location of sampling was defined by the part that was least contaminated with insects and contained few undigested material.
Gloves were used to take the samples and the obtained samples were stored in resealable plastic sample bags in a freezer at -20°C until analysis.

4. *Fecal extraction*

The fecal extraction was performed in the laboratory of the KK Open Zoo, following instructions of the Wildlife Endocrine Manual [Brown, 2008]. For details about extraction reagents, please see Appendix C. First the samples were fully dried in a hot air oven (Memmert GmbH+Co.KG Model 400) at 60°C until the weight no longer changed, which took 4-6 days. The dried feces was pulverized and from each sample an amount of 0,1 +/- 0,01 gram was weighed in glass tubes. The exact weight was noted.
To each glass tube 5 ml of 90% EtOH was added and the tubes were vortexed for 30 seconds. The samples were then boiled in a boiling water bath (Memmert Type WB29) at 96°C for 20 minutes. Preventing the samples from boiling dry, 90% EtOH was added as needed. After the 20 minutes boiling, the fluid amount of every tube was equalized by adding 90% EtOH and the tubes were centrifuged for 20 minutes (acc. 6, RCF 4180xG; Hermle Labortechnik GmbH Type Z206A).
After centrifugation, the supernatant was deposited in a second set of glass tubes, which was placed in the boiling water bath to boil dry.
To the fecal pellets in the first set of glass tubes, 5ml of 90% EtOH was added. It was vortexed for 30 seconds and then centrifuged for 15 minutes (acc. 6, RCF 4180xG). After centrifugation, the extracts of the first set of glass tubes were added to the second set of tubes, already containing the boiled dry first extracts.
The second set of glass tubes was placed in the boiling water bath again to dry. After drying, 1 ml of MeOH was added to each tube and each tube was vortexed for 15 seconds. The glass tubes were dried again and 1ml of dilution buffer was added to each tube. They were vortexed for 30 seconds. The fluid containing the extracts was transferred into 1ml tubes (Eppendorf) and stored by -20°C, until final analysis.

5. *Hormone analysis*

Fecal progesterone was measured with an enzyme immunoassay (EIA), using a single antibody technique, described by Brown et al. in 'Wildlife Endocrine Manual' [2008].
Briefly, the fecal progesterone was measured with an EIA that used progesterone (4-pregnene-3,20-dione; Sigma Diagnostics Cat. # P 0130) as standard. The EIA's were performed in microtitre plates (NUNC Maxisorb) coated with diluted monoclonal progestogen antibody (Quidel clone no. 425; C. Munro, UC Davis, CA). The antibodies were raised against 4-pregnen-11-ol-3,20-dione hemisuccinate:BSA and cross-reacts with various fecal progesterone metabolites [Graham et al, 2001], see appendix A. The label used was diluted 4-pregnene-3,20-dione-3-[O-Carboxymethyl]Oxime-horseradisch peroxidase (progesterone-3CMO-HRP).
The optical density was read at 405nm (Tecan Austria GmbH, model Sunrise – Basic Tecan).
The inter-assay and intra-assay coefficients of variation are respectively 3,7% and 2,75%.
For further details regarding the complete assay protocol, please see Appendix B.

6. *Data analysis*

Definition of the estrous cycle was based on fecal progesterone profiles. Fecal progesterone concentrations are noted as mean ng/g dry feces. To evaluate the length of luteal and follicular phases, the calculation method of Brown et al. [2001] was used. In this method a nonpregnant baseline progesterone value was calculated for each female, using an iterative process in which values that exceeded the mean plus 1.5 standard deviations (SD) were excluded. The average was then recalculated and the elimination process was repeated until no values exceeded the mean plus 1.5 SD. Onset of the luteal phase was defined as the first point after values increased above the baseline by 50% and remained elevated for at least 2 consecutive weeks. The end of the luteal phase was defined as the first of two consecutive values that returned to baseline concentrations.
Estrous cycle length was calculated as the beginning of one luteal phase to the beginning of the next. [Brown et al., 2001]
Besides this, behavioral observation was used to contribute to determining the cycle length. The onset of the luteal phase is considered to occur on the day of mating, whereas the cycle length is the time from this day until the next day of mating [Patton et al., 1999].
The gastrointestinal transit time of fecal progesterone is reported to be between 48 and 72 hours in the white rhinoceros [Radcliffe et al., 1997; Patton et al., 1999].
Unfortunately it is impossible to test for significant differences with only two animals included in this study.

Results

Behavioral observations

From 07/10/2011 untill 14/10/2011 both males were able to approach Som Sri without fighting. She let them rest and rub their chins on her back and with mounting she showed standing behavior and she lifted her tail to the side. Also vaginal discharge has been seen during this time. On 14/10/2011 (day 7 of the study) Som Sri mated with both males several times. The day after these matings the males were still able to approach her and to mount, but her standing behavior was already less present. From two days after mating she already showed aggressive behavior towards the males.
On 08/11/2011 (day 32) and 05/12/2011 (day 59) Som Sri mated several times with Ingocy. Five days prior to these matings Som Sri let the males approach her and she let them rest and rub their chins on her back. Two days prior to the matings the males could mount her, without fighting, but succesfull mating did not occur. On the days of mating Som Sri showed urine squirting and curling and lifting of her tail when the males mounted. The days following the matings Som Sri showed the same behavior as after the first mating (day 7).
According to Patton et al [1999] the onset of the luteal phase can be considered to occur on the day of mating and the end on the next mating day. For Som Sri this means that there can be determined two cycles, the first with a length of 25 days (from 14/10/2011 until 08/11/2011) and the second with a length of 27 days (from 08/11/2011 until 05/12/2011).

On 28/10/2011 (day 25 of this study) Kanoon mated several times with both males. Six days prior to the mating the males could approach, without her being aggressive. Four days prior to the mating she let the mailes rest and rub their chins on her back. On day 23 she let the males mount and showed standing behavior. On day 25 she lifted and curled her tail when being mounted. The day following the mating she showed already aggressive behavior when the males approached her.
From 26/11/2011 untill 30/11/2011 affective behavior between Kanoon and the males has been observed. Kanoon allowed the males approach her more close than usual, without being aggressive. During this timespan also mounting was observed several times with standing behavior from Kanoon, but mating has not been seen. Determining the cycle length of Kanoon based on behavioral observations is not possible, while there has been seen only one moment of several matings with Kanoon.

Fecal progesterone profiles

During this study two female white rhinoceroses were monitored from 04/10/2011 until 07/12/2011. The progesterone profiles of Som Sri and Kanoon are shown in fig. 2a and 2b.

Som Sri showed a peak of 3864 ng/g dry feces on day 20 and 31 days later, on day 51, she showed a second peak of 3592 ng/g dry feces. The first peak was preceded by a nadir of 207 ng/g dry feces on day 12, whereafter progesterone levels increased for 8 days. The second peak was preceded by a nadir of 643 ng/g dry feces on day 38. The progesterone levels thereafter were elevated for 13 days. Unfortunately there were two gaps in sampling between day 28 and 36 and between day 38 and 49, which makes it difficult to draw conclusions to. Nadirs were also seen on day 4 (94 ng/g dry feces), day 24 (539 ng/g dry feces) and day 57 (276 ng/g dry feces). Progesterone levels stayed low for 3 days after the latter nadir.
Mating has been observed 5 and 6 days before a nadir in progesterone levels occurred. The first mating has been seen on day 7, which is 5 days before the nadir of day 12. Furthermore mating has been observed 6 days before the hormone nadir of day 38. The third mating took place on day 59 and coincided with the nadir of day 57 and 60.
For calculation of baseline concentrations, the method according to Brown et al. [2001] was used (see 'Materials and methods'). For Som Sri, the progesterone baseline is at 665 ng/g dry feces. The baseline plus 50% of the baseline is set at 998 ng/g dry feces. The onset of the luteal phase is defined as the first point after values increased above 998 ng/g dry feces and remain elevated for at least two consecutive weeks. The end of the luteal phase was defined as the first of two consecutive values that returned to baseline concentrations. Utilizing the method of Brown et al. [2001], the onset of the first studied luteal phase of Som Sri was at day 13, where progesterone concentrations exceeded the baseline with 50% and remained so until day 36, if we discount the hormone level of day 24. Subsequently, hormone levels decrease to baseline on day 38, with

unfortunately only one sample to confirm this diminution. We assume that day 38 can be stated as the end of the luteal phase. Furthermore, from day 49 values exceed the baseline by 50% again.
Patton et al. [1999] stated that when fecal samples were not collected on consecutive days during the transition from luteal to interluteal phase, half of the missing days were assigned to the luteal and half to the interluteal phase. Applying this to the gap between day 38 and day 49, 5,5 days should be allocated to the interluteal and the same amount to the next luteal phase. This means that the interluteal phase started on day 38 and ended on day 43,5 (day 38 plus 5,5 days), which is a interluteal length of 5,5 days. Total cycle length is determined on 25 days (day 13 until day 38) plus 5,5 day, which is a total of 30,5 days.
Furthermore, until day 55 values exceeded the baseline by 50% again, before decreasing. The last sample showed a value above baseline by 50% again. So only one cycle could be detected in Som Sri, based on progesterone levels.

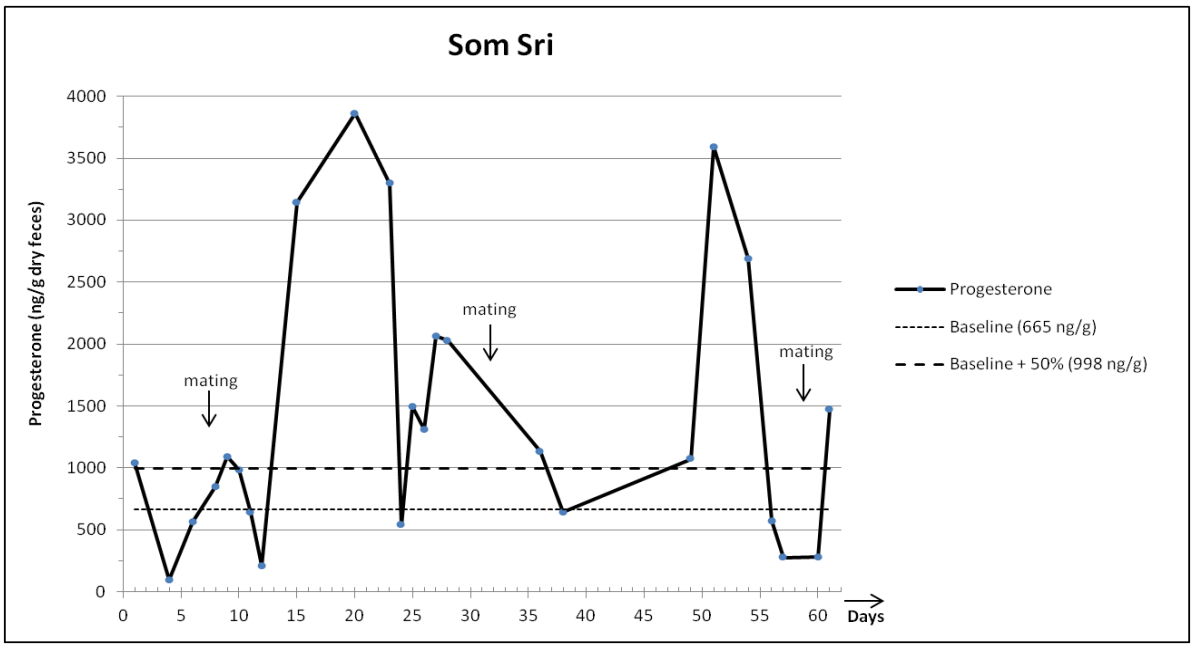

Fig 2a. Fecal progesterone (ng/g dry feces) profile of Som Sri. Collected from 08/10/2011-07/12/2011. Blue dots point out the moments of sampling. Mating occurred on day 7, day 32 and day 59.

The hormone levels of Kanoon showed a peak on day 8 of 4775 ng/g dry feces and there could be expected a peak after day 65 (progesterone level of 3225 ng/g dry feces). The study ended on day 65, so no further details are obtained. One day before the hormonal rise of day 65 a nadir of 133 ng/g dry feces has been detected. On day 18 there was a nadir of 339 ng/g dry feces, which was followed by a rise to 1984 ng/g dry feces on day 19. Thereafter, progesterone levels decreased again to 296 ng/g dry feces on day 28. This latter nadir preceded a fluctuation between day 29 and day 55. Unfortunately, between day 30 and day 54 only one sample was obtained, on day 42.
On day 25, 3 days before the nadir of day 28, mating with Kanoon and both males was observed.
With the method of Brown et al. [2001] Kanoon's baseline is calculated at 439 ng/g dry feces, whereas the baseline plus 50% is 658 ng/g dry feces. From day 1 until day 19 values exceed the baseline by 50%, if we exclude the progesterone level of day 18. Next, there is a decline in hormone concentration to the baseline for about 4 days (day 24 until day 28). Day 29 shows an elevated progesterone level above baseline by 50%, which remains above 658 ng/g dry feces until day 57. Hereafter, there is a decrease below baseline concentrations until day 65 days. Day 65, the last day of sampling, there is noted an increasing hormone level that exceeds the baseline by 50%.
In Kanoon one can determine the luteal phase from day 29 until day 58, which is a length of 29 days. The interluteal phase started on day 58 and lasted until day 65. The cycle length is then calculated on a total of 36 days.

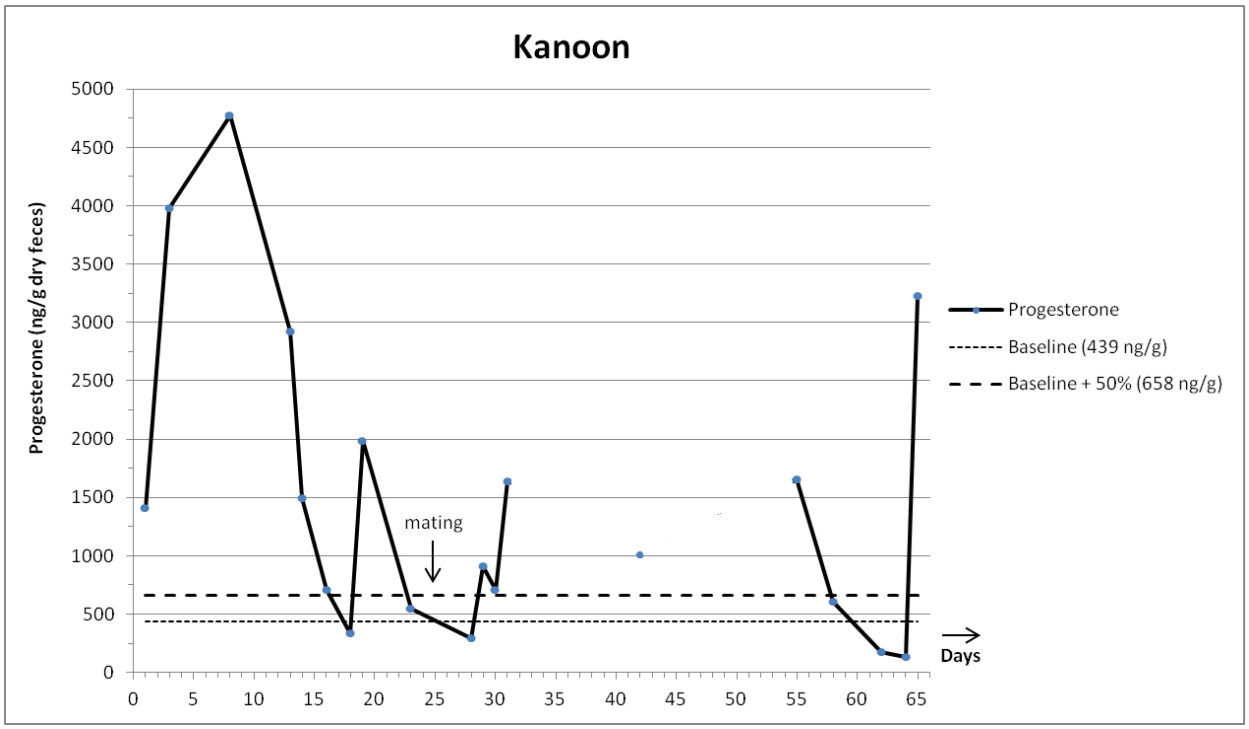

Fig 2b. Fecal progesterone (ng/g dry feces) profile of Kanoon. Collected from 04/10/2011-07/12/2011. Blue dots point out the moments of sampling. Mating occurred on day 25.

Table 3. Cycle lengts based on fecal progesterone profiles.

	Som Sri	Kanoon
Luteal phase	25 days	29 days
Interluteal phase	5,5 days	7 days
Cycle length	30,5 days	36 days

Discussion

The aim of this study was to determine the reproductive status of the two female white rhinoceroses, Som Sri and Kanoon, in the Khao Kheow Open Zoo in Thailand. Fecal samples were used for measuring progesterone concentrations to detect whether the females are cycling. Unfortunately, the short length of the study period and it's regularity of sample collection does not enable to prevents firm conclusions. Therefore, we can only make assumptions regarding the cyclicity of the two female rhinoceroses.
Both progesterone profiles show peak levels and nadirs, which indicate a cyclic pattern. Progesterone increases during luteal phase and is low during estrous [Radcliffe et al., 1997; Schwarzenberger et al., 1998; Patton et al., 1999; Brown et al., 2001; Roth 2006], with ovulation at the nadir of the fall of fecal progesterone concentration [Radcliffe et al., 1997]. Radcliffe et al. [1997] also reported that progesterone concentrations increased 7-9 days after ovulation without conception and remained elevated for 19-20 days.
Som Sri showed a nadir in the fall of hormone levels on day 12, assuming that ovulation occurred that day. After this nadir progesterone concentrations increased to peak level in 8 days, on day 20, which is in accordance with Radcliffe et al. [1997]. Subsequently, the hormone profile showed a nadir on day 38, 18 days after peak level of day 20. This is not fully similar with the 19-20 days stated by Radcliffe et al. [1997], but it is insufficient to draw conclusions about characteristics of the cycle after sampling day 38, as there are no data from day 38 until day 49. The method of Patton et al. [1999] (see Results) to make up which part of the gap in time belongs to the interluteal or the luteal phase is only an estimated guess and hence not fully reliable.
The same applies to Kanoon, whereby there has been seen a nadir on day 28, presumable the day of ovulation. This was followed by an increasing progesterone concentration which remained elevated until day 55. Peak level in this period is not detected, which may be due to two gaps in sampling between day 31 and 42 and between day 42 and 55. Hormone levels seem to be elevated for 27 days subsequently to ovulation, which is in accordance with Radcliffe et al. [1997].

The estimated cycle lengths, based on fecal progesterone profiles, of Som Sri and Kanoon, are respectively 30,5 and 36 days. Other studies analyzing fecal progesterone metabolites also show estrous cycles of approximately 1 month [Radcliffe et al., 1997; Schwarzenberger et al., 1998; Patton et al., 1999; Brown et al., 2001; Carter et al., 2007]. Since it is generally believed that the 30-35 day cycle can be fertile [Radcliffe et al., 1997; Schwarzenberger et al., 1998; Patton et al., 1999], one can conclude that both females could be fertile.
The estimated cycle lengths are somewhat different from the calculated cycle lengths based on behavioral observations. These last lengths might be more reliable, since observation has been done every day, so there are no gaps in time. Only during the night behavior was not observed. This does not mean that reproductive behavior is missed, since courtship and mating behavior can occur at nearly any time of day. [Hutchins and Kreger, 2006]
In Som Sri, three matings were observed, which indicates that she was in estrous three times during the period of this study. The cycle lengths based on these observations are respectively 25 and 27 days. This is quite similar to the cycle length between 26 and 28 days, reported by Morrow et al. [2008], who used fecal progesterone concentrations and observations of courtship behavior.
Only one mating was observed in Kanoon on day 25, but the fecal progesterone profile indicated that there has been a luteal phase at the beginning of the study. It could be possible that she did mate a few days before starting the investigation. When taking this into account, her cycle length would be around 26 days, what could also be in accordance with Morrow et al. [2008]. Since sexual behavior and mounting with Kanoon was observed between day 54 and 58, it could be a possibility that a mating occurred during the nighttime, just before the nadir of day 64 and that it had not been observed, while typical estrous behavior lasts less than one 24 hours-period [Radcliffe et al., 1997].
Patton et al. [1999] found that mating occurred at the nadir of progesterone concentrations, taking in account a lag time of 48-72 hours between actual decrease in blood hormone levels and the fecal measurement of this decrease, due to gastrointestinal transit time. A gastrointestinal transit time of 65 hours has been reported by Radcliffe et al. [1997]. One can estimate that Som Sri's first mating occurred 72 hours prior to a nadir in progesterone levels. The same counts for the mating observed with Kanoon. Taking in account a gastrointestinal transit time of 72 hours, one can conclude that mating in both study females took place on the same day progesterone levels decreased to a nadir, which is consistent with findings of Patton et al. [1999]. Applying this to the second and third mating seen with Som Sri there are respectively 144 and 24 hours between mating and a hormone nadir. However, as mentioned before, it is not possible to draw firm conclusions about these dates, because of a lack in data.
Despite the short timespan of this study and the irregularity of sample collection, one can assume that Som Sri and Kanoon are cycling and do not belong to the approximate 50% of the females that exhibit an acyclic pattern wherein progesterone remains at baseline concentrations for extended periods ranging from several months to years [Schwarzenberger et al., 1998; Patton et al., 1999; Brown et al., 2001; Roth, 2006; Carter et al., 2007]. To determine whether Som Sri and Kanoon exhibit a regular pattern of cycle lengths, a long-term study must be performed. However, one must take into account that reproductive patterns are highly variable among and within individuals [Schwarzenberger et al., 1998]. Differences in cycle length could be due to reproductive pathologies like pyometra or endometritis causing early embryonic loss, which could lead to a prolonged luteal phase and an extended progesterone secretion [Radcliffe et al., 1997; Patton et al., 1999; Brown et al., 2001].
Swaisgood et al. [2006] studied the low reproduction rate in captive-born females (F1) compared with this in founders (F0). He found that F1 females that have mated, are less likely to produce offspring than F0 females do. His most important finding is that F1 females must experience post-copulatory reproductive problems, resulting in failures to conceive or maintain pregnancy. He suggested that this could be caused by an abnormal development of the genital system in captive-born females [Swaisgood et al., 2006]. Since Som Sri and Kanoon are both founders, one should argue that conclusions of Swaisgood et al. [2006] are not applicable. However, these females were 1,5 and 2,5 years old when captured from the wild. This means they had not reached the age of sexual maturity, which is at 6-7 years [International Rhino Foundation, 2011], so their reproductive system was still developing. An abnormality in this development might be a possible explanation for their failure in producing offspring.
To investigate if Som Sri and Kanoon suffer from reproductive abnormalities, additional examination like ultrasonography would be highly valuable to investigate ovulation or pregnancy.
Besides reproductive pathologies, poor breeding performance can be due to management factors, such as enclosure size and herd dynamics, including dominance status [Schwarzenberger et al., 1998]. Patton et al. [1999] hypothesized that female rhinoceroses may require a period of isolation from the male to maintain

sexual responsiveness and perhaps even ovarian function, as non-breeding individuals sometimes become breeder when new opposite-sex animals are introduced. Based on this, one can try to separate Som Sri and Kanoon from the males and introduce them again after a while.
Another factor influencing reproduction, especially in captive rhinoceroses, is stress [Brown et al., 2001]. In this case, stress can be of high importance, because the rhinoceroses live in an enclosure where people can interfere. To find out if stress influences the reproductive performances of Som Sri and Kanoon, my colleague K. de Keyzer [2011], performed a study to determine the influence of corticoid concentrations on the reproductive status simultaneously with this study. A conclusion of K. de Keyzer's study is that cortisol levels of Som Sri and Kanoon are high, but do not seem to interfere with cyclicity. If the high levels of stress can cause early embryonic loss has yet to be determined. For further details, please see the study of K. de Keyzer [2011]. Another cause for the rhinoceroses breeding problem in the Khao Kheow Open Zoo, is that the males suffer from fertility problems, instead of the females.

With this study and the study of K. de Keyzer [2011] we evaluated the breeding problem of Som Sri and Kanoon in the Khao Kheow Open Zoo in Thailand. As mentioned before, the period of this study and regularity of sample collection was not sufficient enough to draw firm conclusions. Still, one can conclude that both female rhinoceroses are not acyclic, but a long-term study for over 2 months with regular sample collection should be performed to gain more data for more comprehensive hormonal profiles. Together with fecal hormonal analysis, one should perform ultrasonographic examination, if practically and financially feasible, to obtain more information about the reproductive failure in Som Sri and Kanoon.

Conclusion

1. Measuring fecal progesterone together with observation of reproductive behavior is a good non-invase method to get information about the reproductive status of female southern white rhinoceroses.
2. Som Sri and Kanoon seem to be cyclic and show estrous behavior.
3. A long-term study period with regular sample collection is necessary to elucidate the breeding problem of Som Sri and Kanoon.
4. Adding the use of ultrasonography at the study will help to obtain valuable information about the causes of poor breeding performance.

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Appendix A

Cross-reactivity of the Quidel clone no. 425 gen antibody to various progesterone metabolites (relative to the binding of progesterone) [Graham et al., 2001]

Progesterone metabolite	Common name	Cross-reactivity (%)
4-Pregnen-3,20-dione	Progesterone	100.0
4-Pregnen-3α-ol-20-one		188.0
4-Pregnen-3β–ol-20-one		172.0
4-Pregnen-11α-ol-3,20-dione		147.0
5α-Pregnan-3β–ol-20-one		94.0
5α-Pregnan-3α–ol-20-one		64.0
5α-Pregnan-3,20-dione		55.0
5β-Pregnan-3β-ol-20-one		12.5
5β-Pregnen-3,20-dione		8.0
4-Pregnen-11β-ol-3,20-dione		2.7
5β-Pregnan-3α-ol-20-one		2.5
5β-Pregnan-3α,20α-diol	Pregnanediol	<0.1
5α-Pregnan-3α,20β-diol		<0.1
5β-Pregnan-3,17-dione	Androstenedione	<0.1
5β-Pregnan-11β,21-diol-3,20-dione	Corticosterone	<0.1

Appendix B

Condition scoring system by Reuter and Adcock [1998]

Modified body condition scoring system by Reuter and Adcock [1998]

	CONDITION Assessment site	Numerical scale Descriptive scale	5 excellent (heavy)	4 good (ideal)	3 fair (average)	2 poor (thin)	1 very poor (emaciated)
A	Neck	General appearance	thick, well muscled, rounded	well muscled, rounded	rounded	flat, narrow neck; nuchal ligament visible	narrow, angular (bony) neck; nuchal ligament prominent
		Prescapular groove		-	slightly visible	obvious	deep groove very obvious
B	Shoulder	General appearance	well-muscled, rounded	rounded	flat	flat, slightly angular (bony)	angular, bony
		Scapula	covered	covered	spine visible	obvious	very obvious
C	Ribs		well covered (skin folds)	covered (skin folds)	visible	obvious	very obvious
D	Spine	General appearance	rounded	slightly angular	back groove back visible	groove deep obvious	back groove very obvious
		Spinous processes	covered	slightly visible	visible	prominent	very prominent
E	Rump	General appearance	well rounded	flattened	slightly concave	concaveobvious depression	
		Bony protuberances	covered	slightly visible	visible	prominent	very prominent
F	Abdomen	General appearance	distended, taught	filled	slightly tucked in	tucked in	tucked in
		Flank-fold	none	sometimes slightly visible	slightly visible	visible	obvious
G	Tail base		rounded (bulging)	rounded	narrow	slightly bony	very thin and bony

Fig 3a. The body regions and specific anatomical features to be observed when assessing a rhino's condition [Reuter and Adcock, 1998]

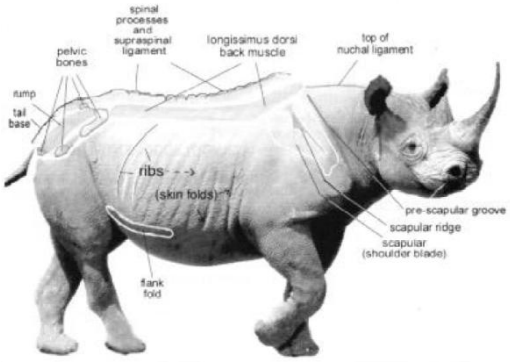
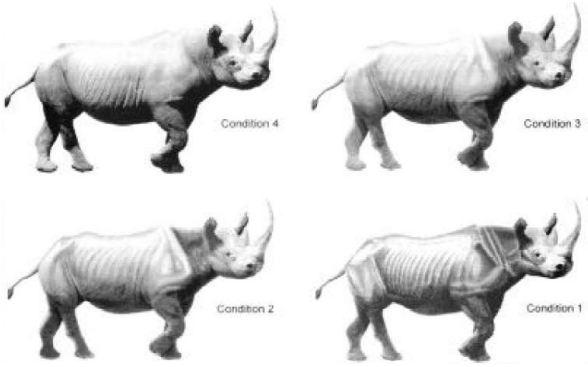


Fig 3b. The appearance of black rhino for all body condition scores. [Reuter and Adcock, 1998]



Appendix C

Assay protocol progesterone (CL425) EIA per plate – by Brown et al. [2008]

Day 1

1. Plate Coating
   - Use NUNC Maxisorb plates.
   - Add 20 µl antibody stock (1:50, -20°C) to 5 ml coating buffer (working dilution 1:10,000).
   - Add 50 µl per well of antibody solution to plate.
   - Do not coat column 1 - start at A2 and go down each column.
   - Pipet all solutions in this order.
   - Tap plates gently to ensure that coating solution covers bottom of well.
   - Label, cover with acetate plate sealer and leave overnight (no less than 12 hrs) at 4°C.

Day 2

2. Standards
   - Standard values used are: 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 pg/well.
   - Dilute standard working stock (4 ng/ml or 200 pg/well) serially (2-fold) by using 200 µl stock plus 200µl EIA buffer.
3. Samples/controls
   - Dilute urine or fecal samples in dilution buffer to appropriate dilution. (1:128)
   - Prepare High and Low controls.
4. HRP
   - Progesterone HRP working dilution is 1:40,000.
   - Add 23 µl of HRP working stock to 6 ml EIA buffer to make the working dilution (keep this solution cool).
5. Plate washing
   - Wash the plate five times with wash solution.
   - Blot the plate on paper towel to remove excess wash solution.
6. Plate loading
   - Pipet 50 µl of standard, control and samples per well as quickly and accurately as possible.
   - Add 50 µl of diluted Progesterone HRP (step 4) to all wells that contain standard, control, or sample. Avoid splashing.
   - No more than 10 minutes should pass during this process.
   - Cover plates with acetate plate sealer and incubate at room temperature for exactly 2 hours.
7. Plate washing
   - Wash the plate five times with wash solution.
   - Blot the plate on paper towel to remove excess wash solution.
   - Plates are fairly stable at this point and can be left upside down on bench top until all plates are washed (no more than 1 hour).
8. Substrate
   - Prepare ABTS substrate immediately before use (within 20 min).
   - Combine 40 µl 0.5 M H_2O_2, 125 µl 40 mM ABTS and 12.5 ml substrate buffer and mix well.
   - Add 100 µl ABTS substrate to all wells that contain standard, control, or sample.
   - Cover with plate sealer and incubate at room temperature for 40 minutes.
9. Plate reading
   - Read at 405 nm (reference 540 nm).

Progesterone stock preparation – per plate – by Brown et al. [2008]

4. Antibody
- Dilute monoclonal CL425 at a dilution of 1:50 by adding 100 µl of stock to 4.9 ml of coating buffer.
- Aliquot 300-400 µl into O-ring vials and store at -20ºC.
- Store Antibody stock at -80ºC.

2. HRP Conjugate
- Dilute progesterone-3CMO-horseradish peroxidase 1:200 by adding 25 µl of stock to 4.975 ml EIA buffer for a working stock and store at 4ºC.
- Store HRP stock at -80ºC.

3. Standards
- Weigh out 0.5 mg progesterone (Sigma Diagnostics Cat. # P 0130) and add to 5 ml ETOH in a scintillation vial for a 100 µg/ml primary stock solution
- Dilute 100 µg/ml (100 000 ng/ml) primary stock 1:100 by adding 40 µl to 4 ml ETOH for a 100 ng/ml secondary stock.
- Dilute 100 ng/ml secondary stock 1:2500 by adding 200 µl to 49.8 ml of EIA Buffer for a 4 ng/ml (200 pg/well*) working stock.
- Aliquot working stock and store all stocks at -20ºC.

*a well is equal to 50 µl, the amount used in the assay.

4. Controls
- Use urine or extracted fecal samples with a high progestin levels to make controls.
- Make a pool of high progestin level urine or extracted feces (~20 ml).
- Serially dilute pool and run on assay.
- Find the dilutions that bind at ~70% and ~30%.
- Use the pool to make up two separate stocks for low and high controls using the dilutions that bound at 70% and 30% respectively.
- Make up enough controls to run on at least 500 assays (for this you may need more than the 20 ml used for the pool. Any species will do so long at the urine has high progestin levels).

Assay reagents and supplies – by Brown et al. [2008]

1. STEROID EIA ASSAY RECIPES (full and half recipes)

COATING BUFFER	Full	Half	
Na_2CO_3 (Anhydrous)	1.59 g	0.795 g	(Sigma, S2127)
$NaHCO_3$	2.93 g	1.465 g	(Sigma, S8875)
H_2O	1000 ml	500 ml	

pH to 9.6, store at 4°C

STOCK A			
Stock A 0.2M NaH_2PO_4	27.8 g	13.9 g	(Sigma, S9638)
H_2O	1000 ml	500 ml	

STOCK B			
Stock B 0.2M Na_2HPO_4	28.4 g	14.2 g	(Sigma, S0876)
H_2O	1000 ml	500 ml	

ASSAY BUFFER (EIA BUFFER)			
Stock A	195 ml	97.5 ml	
Stock B	305 ml	152.5 ml	
NaCl	8.7 g	4.35 g	(Sigma, S9625)
BSA	1.0 g	0.5 g	(Sigma, A7906)
H_2O	500 ml	250 ml	

pH to 7.0, store at 4°C

"Dilution buffer" for fecal extraction is EIA buffer minus the addition of BSA.

WASH SOLUTION STOCK (*dilute before using)			
NaCl	87.66 g	43.83 g	(Sigma, S9625)
Tween 20	5.0 ml	2.5 ml	(Sigma, P1379)
H_2O	1000 ml	500 ml	

store at 4°C

*Dilute 10-fold for working wash soln. (i.e.100 ml wash concentrate plus 900 ml H_2O), store at RT

SUBSTRATE BUFFER			
Citric acid (anhydrous)	9.61 g	4.805 g	(Sigma, C0759)
H_2O	1000 ml	500 ml	

pH to 4.0, store at 4°C

ABTS (40 mM)		
ABTS	0.55 g	(Sigma, A1888)
H_2O	25 ml	

pH to 6.0. ABTS is light sensitive - use brown glass or foil for storage
store at 4°C

HYDROGEN PEROXIDE (0.5M)		
H_2O_2 (30% Solution)	500 µl	(Sigma, H1009)
H_2O	8 ml	

store at 4°C