

## Faecal progesterone metabolite analysis for non-invasive monitoring of reproductive function in the white rhinoceros (*Ceratotherium simum*)

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### Abstract

The two subspecies of white rhinoceros, southern (*Ceratotherium simum simum*) and northern (*Ceratotherium simum cottoni*), breed poorly in captivity, and estimates of oestrous cycle length vary considerably (range, 25–90 days). To characterise reproductive patterns, faecal samples were collected 2–3 times/week for up to 56 months from non-pregnant animals ( $n = 21$ ) of both subspecies. Immunoreactive pregnanes containing a 20-oxo-group (20-oxo-P) were analysed in a group-specific enzymeimmunoassay using an antibody against 5 $\alpha$ -pregnane-3 $\beta$ -ol-20-one 3HS:BSA. Reproductive patterns were highly variable among and within individual animals. However, rhinoceroses could be classified into four major categories on the basis of oestrous cycle length and luteal phase 20-oxo-P concentrations: (1) regular oestrous cycles of 10 weeks duration and  $> 800$  ng/g ( $n = 2$  animals); (2) oestrous cycles between 4–10 weeks and 250–750 ng/g ( $n = 6$ ); (3) no apparent cycle regularity, but luteal activity indicated by 20-oxo-P concentrations of 100–200 ng/g ( $n = 6$ ); (4) no apparent luteal activity as indicated by 20-oxo-P of  $< 100$  ng/g ( $n = 7$ ). In two attempts to induce ovarian activity, chlormadinone acetate was fed daily to one animal for 35 and 45 days, respectively. Each treatment was followed by a subsequent hCG injection which resulted in luteal phases of 17 and 18 days, respectively, beginning about 10 days

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after hCG. Concentration of faecal 20-oxo-P in one pregnant animal during the 4th and 5th month of gestation were markedly higher than those observed during the luteal phase of the cycle. In conclusion, two thirds of white rhinoceroses in this study had erratic or missing luteal activity, whereas variable cycles of 4–10 weeks in length were evident in six females, and regular oestrous cycles of 10 weeks in length were found in two animals. © 1998 Elsevier Science B.V. All rights reserved.

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## 1. Introduction

The white rhinoceros (*Ceratotherium simum*) exists in two subspecies, the northern (*Ceratotherium simum cottoni*) and the southern (*Ceratotherium simum simum*) white rhinoceros. White rhinoceroses are territorial and feed by grazing. In the wild, adult females are usually only accompanied by a single offspring. Subadults tend to associate in pairs and bulls typically are solitary. If a bull remains with a cow for more than a day, it is assumed the cow is coming into oestrus (Owen-Smith, 1988; Pienaar, 1994). The numbers of southern white rhinoceros are ~ 7500 animals in the wild (Foose, 1997) and ~ 700 animals in captivity (Ochs, 1995), making it more abundant than any of the other surviving rhinoceros species: black (*Diceros bicornis*), Indian (*Rhinoceros unicornis*), Sumatran (*Dicerorhinus sumatrensis*) and Javan (*Rhinoceros sondaicus*) combined. However, poaching pressure is still intense and almost all southern, free-ranging white rhinoceroses are confined to the Republic of South Africa. Hence, one cannot be complacent about their conservation (Foose, 1997). In contrast to the southern subspecies, the northern white rhinoceros is among the most endangered, consisting of only 29 surviving animals in the Garamba National Park in Zaire and 12 animals in captivity (Foose, 1997).

In contrast to wild white rhinoceroses which are increasing at an annual rate of 8–9% (Owen-Smith, 1988), the captive population of both subspecies is in a demographic crisis. As of December 1994, 54% of captive southern white rhinoceroses were > 20 years of age, and the reproductive rate of founders and first and second generation offspring were only 30%, 8% and 0%, respectively (Ochs, 1995). The higher reproduction rate in founders was largely due to the import of pregnant animals from the wild. Reproduction in captive northern white rhinoceroses is even worse; only one female has ever reproduced, and she is now deceased. The reasons for this poor breeding performance are unclear. Reproduction is influenced by management factors such as enclosure size and herd dynamics, including dominance status (Mikulica, 1991; Boer and Hamza, 1996). Although the average group size of rhinoceroses in South Africa is only 2.1–2.3 animals (Owen-Smith, 1988), reproduction in the captive populations is greatest in zoos that have large enclosures and group sizes of > 2 animals.

Limited information is available on the reproductive physiology of white rhinoceroses, and the data that do exist are conflicting, especially with regards to the oestrous cycle. Behavioural observations of captive white rhinoceroses indicated oestrous cycle lengths ranging from 30–90 days (Lindemann, 1982; Boer and Hamza, 1996), whereas Owen-

Smith (1988) reported an oestrous cycle length of about 30 days for animals in South Africa. On the basis of rectal palpation, vaginal cytology and urinary steroid analysis, Wagner (1986) concluded the oestrous cycle length was 42 days. Another study using urinary steroid analysis indicated the oestrous cycle was 25 and 32 days in lengths for the northern and southern subspecies, respectively (Hindle et al., 1992). Finally, a recent study by Radcliffe et al. (1997) used combined ultrasonography and faecal progesterone metabolite monitoring in one white rhinoceros and described an initial period of ~3 months in which there was some ovarian activity, but no ovulation, followed by two non-conceptive cycles (31–35 days in length) and two conceptive cycles which ended in early embryonic death after day 28. The conceptive periods were 73–78 days in length, and the luteal structures persisted 42–48 days.

The objectives of the present study were to validate group-specific enzymeimmunoassays for measuring faecal immunoreactive pregnanes containing a 20-oxo-group (20-oxo-P) in the northern and southern white rhinoceros, and to use this approach to: (1) characterise the oestrous cycle; (2) evaluate the efficacy of hormonal treatment to induce ovulation in a non-cycling female; and (3) assess luteal activity during pregnancy (months 4 and 5).

## 2. Materials and methods

### 2.1. *Animals and sample collection*

Twenty-one captive white rhinoceroses (16 southern, four northern and one hybrid) were studied (Table 1). Zoos with more than one female managed them in groups. In general, females were housed indoors at night in separate pens, but were together with a male during the day in outdoor enclosures. During the summer, and depending on weather conditions, animals from Dvur Kralove were left outside at night, together with a bull. The outdoor enclosure was cleaned in the morning, and, at that time, animals were placed in indoor pens where faecal samples were collected. Likwezi from Melbourne/Werribee was together with the male at all times (day and night). These animals were fed in a corral in the mornings, where samples from Likwezi were collected after observed defecation. Group compositions (male/female) were: Arnheim Zoo 1.1; Arnheim Safari 5.1 and 4.1 in 1994 and 1995, respectively; Berlin Tierpark 2.3 (of which 1.1 were subadults); Melbourne/Werribee 1.1; Salzburg 2.2; Toronto 2.2 (of which 1.0 was subadult); Usti nad Labem 3.2 (of which 2.0 were subadults); Dvur Kralove: 1.1 for Sanni; and 5.1 in 1992 and 4.1 from 1993 onwards for animals from the northern subspecies, which were housed together with the hybrid female. Oestrous behaviour and mating was recorded by keeper staff.

Faecal samples were usually collected 2–3 times/week; however, samples from Likwezi, and from Pistol and Shaboola in 1994 were available about once per week. Freshly defecated samples were collected in the morning over periods of 6–56 months (with the exception of one animal from which samples were collected for only three months). Additionally, samples from one pregnant animal were collected during months 4 and 5 of gestation. Samples were frozen immediately after collection at  $-20^{\circ}\text{C}$  and shipped to Vienna where they were stored at  $-20^{\circ}\text{C}$  until analysis.

Table 1  
Rhinos investigated in this study

Subspecies	Location	Studbook number	Birth date	Recent deliveries	Samples collected	Collection period (months)	Category of luteal activity <sup>a</sup>
<i>Ceratotherium simum sinum</i>							
Inneke	Arnheim Zoo	171	1969	–	5–9/95	3	2
Ghea	Arnheim Safari	174	1969	1977;1981 1984;1988	6–10/94; 5–9/95	10	4
Rhulji	Arnheim Safari	175	1969	–	6–10/94; 5–9/95	10	2
Freya	Arnheim Safari	230	1971	1979	6–10/94; 5–9/95	10	2
Petra	Arnheim Safari	643	1981	–	6–10/94; 5–9/95	10	2
Maya	Arnheim Safari	840	1984	–	6–10/94	5	4
Lotzi	Berlin Tierpark	179	1971	–	4–10/96	6	3
Kifaru	Berlin Tierpark	773	1982	–	4–10/96	6	3
Sanni	Dvur Kralove	199	1966	–	4–10/92	7	4
Likwezi	Meibourne Werribee	871	1985	–	6/94–1/95	2	2
Baby	Salzburg	361	1971	–	10/91–10/96	56	4
Kathy	Salzburg	362	1972	–	10/91–10/96	56	1 + 2
Pistol	Toronto	218	1973	1979;1982 1985;1990	8/94–5/96	21	1
Shaboola	Toronto	582	1979	–	8/94–5/96	21	2 + 4
Sasa	Usti nad Labem	114	1967	1986;1991 Dec 1993	12/92–1/93; 5/94–5/95	14.5	3
Zamba	Usti nad Labem	209	1970	–	5/94–5/95	13	3
<i>Ceratotherium simum cottoni</i>							
Nasima	Dvur Kralove	351	1965	1977;1980 1983;1989	2–6/92	4.5	4
Nesari	Dvur Kralove	377	1972	–	4/92–4/96	44	4 + 2
Nabire	Dvur Kralove	789	1983	–	2/92–4/96	39	(2) 4
Najin	Dvur Kralove	943	1989	–	1/94–4/96	27	3
<i>C. sinum sinum</i> × <i>C. sinum cottoni</i>							
Nasi	Dvur Kralove	476	1977	–	4/92–4/96	39	(4) 3

<sup>a</sup> For definition of categories, see Section 3. If two numbers are given, the first or the number without parentheses defines the predominant type of category.

## 2.2. Faecal extraction

Faeces (0.5 g) were extracted with methanol as described by Schwarzenberger et al. (1996b) for the black rhinoceros. Briefly, 0.5 g of wet faeces were mixed with 1.0 g of powdered aluminium oxide, 0.5 ml of water and 4 ml of methanol. Samples were vortexed for 30 min and then defatted by vortexing for 10 s in 3 ml of petroleum ether. The methanol fraction was transferred into a new vial, diluted in buffer and analysed using enzymeimmunoassays (EIA).

## 2.3. Enzymeimmunoassays

Faecal extracts were analysed with two group-specific EIAs as described previously for the black rhinoceros (*D. bicornis*, Schwarzenberger et al., 1996b). Briefly, the antibodies were raised in rabbits against 5 $\alpha$ -pregnane-3 $\beta$ -ol-20-one 3HS:BSA and 5 $\beta$ -pregnane-3 $\alpha$ -ol-20-one 3HS:BSA, respectively. Antibodies were group-specific, measuring progesterone metabolites containing a 20-oxo-group, and the results were considered measurements of total immunoreactive 20-oxo-pregnanes (20-oxo-P).

Significant cross-reactivities in the assay using the 5 $\alpha$ -pregnane-3 $\beta$ -ol-20-one 3HS:BSA antibody were: 4-pregnen-3 $\alpha$ -ol-20-one (390%), 5 $\alpha$ -pregnane-3,20-dione (168%), progesterone (100%), 5 $\alpha$ -pregnane-3 $\alpha$ -ol-20-one (89%), 5 $\beta$ -pregnane-3 $\alpha$ -ol-20-one (88%) and 5 $\alpha$ -pregnane-3 $\beta$ -ol-20-one (56%). The assay using the 5 $\beta$ -pregnane-3 $\alpha$ -ol-20-one EIA cross-reacted with 5 $\beta$ -pregnane-3,20-dione (151%), 5 $\alpha$ -pregnane-3 $\beta$ -ol-20-one (102%), 4-pregnene-3,20-dione (progesterone, 100%), 5 $\alpha$ -pregnane-3,20-dione (75%), 5 $\beta$ -pregnane-3 $\beta$ -ol-20-one (36%), 5 $\beta$ -pregnane-3 $\alpha$ -ol-20-one (20%) and 5 $\alpha$ -pregnane-3 $\alpha$ -ol-20-one (8%). Progesterone standard and biotinylated labels were used in both EIAs. Assays were validated by demonstrating parallelism between standard curves and serial dilution of faecal extracts. The intra- and inter-assay coefficients of variation for the assay using the 5 $\alpha$ -pregnane antibody were 11.3% and 16.2%, respectively, and 9.6% and 14.6% for the assay using the 5 $\beta$ -pregnane antibody.

## 2.4. Within sample variation in faecal 20-oxo-P concentrations

Considerable differences in luteal phase concentrations of faecal 20-oxo-P values were observed among animals. In vivo radiolabel infusion studies have suggested that progesterone metabolites might be unevenly distributed in the faeces, with higher concentrations in the outer layer (Palme et al., 1996; Wasser et al., 1996). To determine the within sample variation in faecal steroid concentrations, sample aliquots from the outer layer and centre portion of freshly-defecated faecal balls from one individual (Kathy from Salzburg Zoo) were collected over a 4.5 month period.

## 2.5. High performance liquid chromatography (HPLC) of faecal extracts

To determine assay specificity and to obtain indications on the possible structure of immunoreactive progesterone metabolites, extracts of faecal samples collected during the follicular and mid-luteal phase of the rhino 'Kathy' (samples from April and May 1993) were separated on a straight-phase HPLC system, similar to that described

previously (Schwarzenberger et al., 1996b). Samples were mixed with [ $^3\text{H}$ ]progesterone and [ $^3\text{H}$ ]-20 $\alpha$ -dihydroprogesterone, extracted and separated by HPLC using a solvent gradient of methanol in *n*-hexane/chloroform (75/25 v/v). Fractions were analysed in both of the EIAs. The HPLC elution profiles of the immunoreactive faecal steroids were compared with those of [ $^3\text{H}$ ]progesterone and [ $^3\text{H}$ ]-20 $\alpha$ -dihydroprogesterone and with different 5 $\alpha$ - and 5 $\beta$ -pregnanes containing a 20-oxo-group which cross-reacted in both EIAs. In general, pregnanediones (5 $\alpha$ /5 $\beta$ -pregnane-3,20-dione) eluted before [ $^3\text{H}$ ]progesterone, while monohydroxylated 20-oxo-pregnanes (5 $\alpha$ - and 5 $\beta$ -pregnane-3 $\alpha$ /3 $\beta$ -ol-20-one) eluted between [ $^3\text{H}$ ]progesterone and [ $^3\text{H}$ ]-20 $\alpha$ -dihydroprogesterone.

## 2.6. Protocol for ovulation induction

On the basis of faecal 20-oxo-P analysis, the female 'Baby' from Salzburg Zoo had no luteal activity in > 2 years. Therefore she was treated with the synthetic progestin chlormadinone acetate (CMA; Synchrosin<sup>®</sup>, Werfft-Syntex, Vienna), followed by an hCG injection (Chorulon<sup>®</sup>, Intervet, Boxmeer) to induce ovarian activity. The dose and treatment interval were calculated allometrically (Sedgwick, 1993) using the horse as a model and the following assumptions/estimations: (1) body weight was 2500 kg; (2) the constant (K) for the calculation of Minimum Energy Cost was 70; and (3) the normal cycle length was 10 weeks (based on cycle length of herd mate Kathy). A total of 32 doses of 35 mg of CMA (0.014 mg/kg body weight) each were fed at 36 h intervals for 45 days. Five days after the last CMA treatment, 8400 IU hCG was given in a single intramuscular injection. This treatment regimen was applied twice ~ 9 months apart; however, during the second treatment period CMA was given only for 35 days.

## 2.7. Data analysis

Data are presented as mean  $\pm$  SEM. Definition of the follicular (FP) and luteal (LP) phases of the reproductive cycle was based on faecal 20-oxo-P values. Onset of the LP was defined as the first point after values had increased by > 50 ng/g and remained at > 120 ng/g of faeces for at least two consecutive values. The end of the LP was defined as the first of two consecutive values that were less than 120 ng/g faeces. Because samples were not collected every day, the interval between the consecutive values of the end of the FP and the beginning of the LP was divided by 2 and resulting values added to the FP and the LP, respectively. Oestrous cycle length was calculated from the beginning of one LP to the beginning of the next. The coefficient of correlation was calculated between the concentrations of faecal 20-oxo-P measured in the outer layer vs. the centre portion of the faecal ball, and between the two EIAs used.

# 3. Results

## 3.1. HPLC separation of faecal 20-oxo-pregnanes

Elution profiles between FP and LP samples were comparable, qualitatively, but not quantitatively. During the LP, four major immunoreactive 20-oxo-P peaks were de-

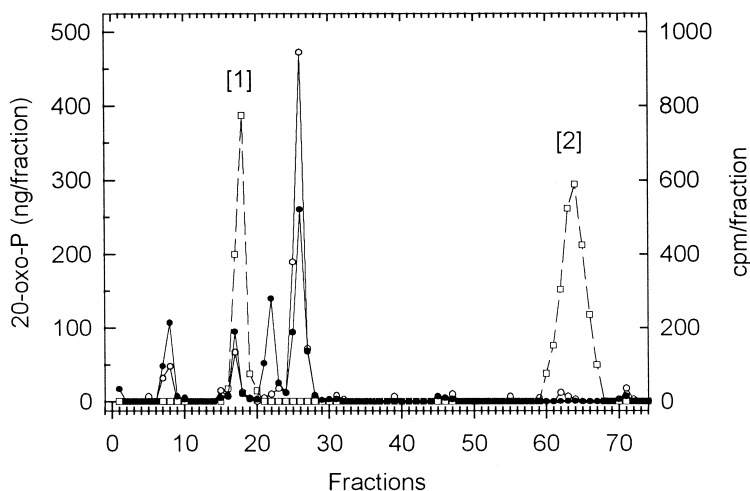


Fig. 1. HPLC separation of immunoreactive 20-oxo-P in a faecal sample collected during the luteal phase of a white rhinoceros. Elution patterns of [ $^3\text{H}$ ]progesterone [1] and [ $^3\text{H}$ ]-20 $\alpha$ -dihydroprogesterone [2] were determined by liquid scintillation counting ( $\square$  cpm/fraction). Fractions were analysed with two group-specific EIAs for 20-oxo-pregnanes which used antibodies against 5 $\alpha$ -pregnane-3 $\beta$ -ol-20-one 3HS:BSA ( $\bullet$ ) and 5 $\beta$ -pregnane-3 $\alpha$ -ol-20-one 3HS:BSA ( $\circ$ ), respectively. Concentrations in ng/fraction were calculated for 1 g of faeces without correction for methodological losses.

tected, one of which coeluted with [ $^3\text{H}$ ]progesterone (Fig. 1). Comparison with corresponding reference steroids together with the different cross-reactivities in the two EIAs indicated that the faecal immunoreactive peaks not coeluting with [ $^3\text{H}$ ]progesterone are

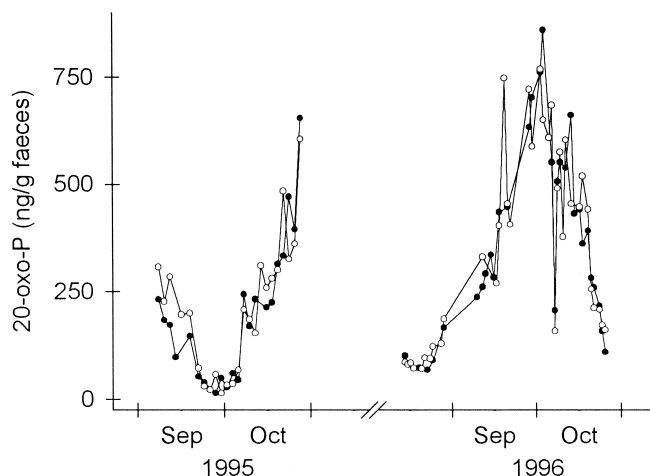
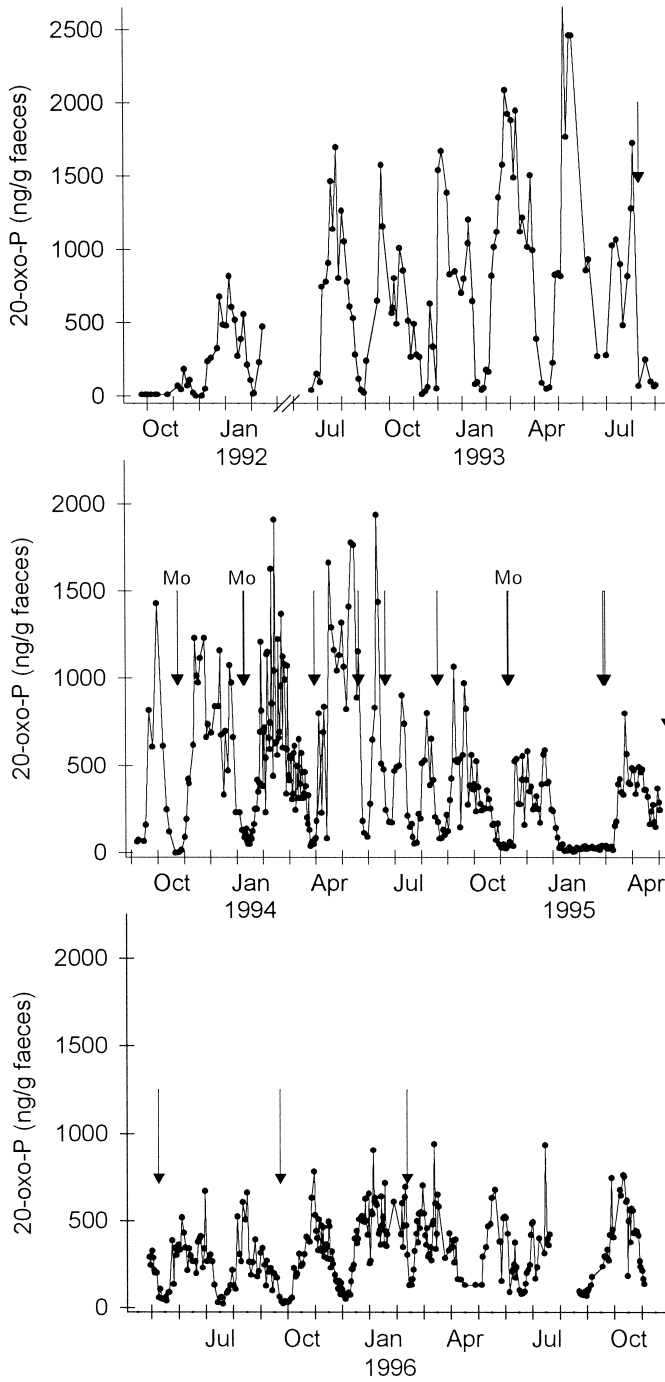


Fig. 2. Variation of 20-oxo-P within faecal balls of a white rhinoceros. Samples were collected from the outer layer ( $\circ$ ) and the centre portion ( $\bullet$ ) of the faecal balls and analysed in an EIA using an antibody against 5 $\alpha$ -pregnane-3 $\beta$ -ol-20-one 3HS:BSA.





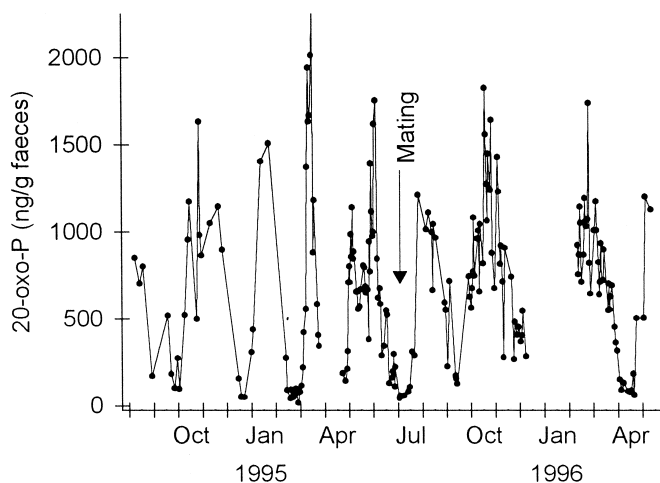


Fig. 4. Concentrations of faecal 20-oxo-P in a white rhinoceros (Pistol) which, according to the regularity of oestrous cycles and luteal phase 20-oxo-P concentrations, was classified as category 1.

the following  $5\alpha$ -reduced pregnanes:  $5\alpha$ -pregnane-3,20-dione,  $5\alpha$ -pregnane-3 $\alpha$ -ol-20-one and  $5\alpha$ -pregnane-3 $\beta$ -ol-20-one, which eluted in fractions 7–8, 21–23 and 25–27, respectively.

### 3.2. Faecal 20-oxo-P profiles

Concentrations of faecal 20-oxo-P were similar between the two antibodies tested ( $r = 0.85$ ;  $P < 0.001$ ); therefore, only results of the EIA using the antibody against  $5\alpha$ -pregnane-3 $\beta$ -ol-20-one 3HS:BSA are shown. The concentration of 20-oxo-P within the faeces did not differ significantly between the outer layer and the central portion of the faecal balls ( $r = 0.80$ ;  $P < 0.001$ ; Fig. 2). Therefore, mean values for the respective days were calculated and used in Fig. 3.

Oestrous cycle characteristics and LP 20-oxo-P concentrations varied among and, to a certain extent, within animals. In general, white rhinoceroses could be classified into four categories (Table 1; Figs. 3 and 5–7) on the basis of oestrous cycle length and LP 20-oxo-P concentrations: (1) females with regular,  $\sim 10$  weeks oestrous cycles and high luteal phase 20-oxo-P concentrations ( $> 800$  ng/g of faeces); (2) females with oestrous cycles ranging in length from 4–10 weeks with luteal phase 20-oxo-P concentrations of

Fig. 3. Concentrations of faecal 20-oxo-P in a white rhinoceros (Kathy) during 1991–1996. According to the regularity of oestrous cycles and luteal phase 20-oxo-P concentrations, oestrous cycles in this female were generally classified as category 1 until May 1994, and as category 2 after June 1994. Arrows indicate behavioural oestrous, Mo indicates mounting by the bull.



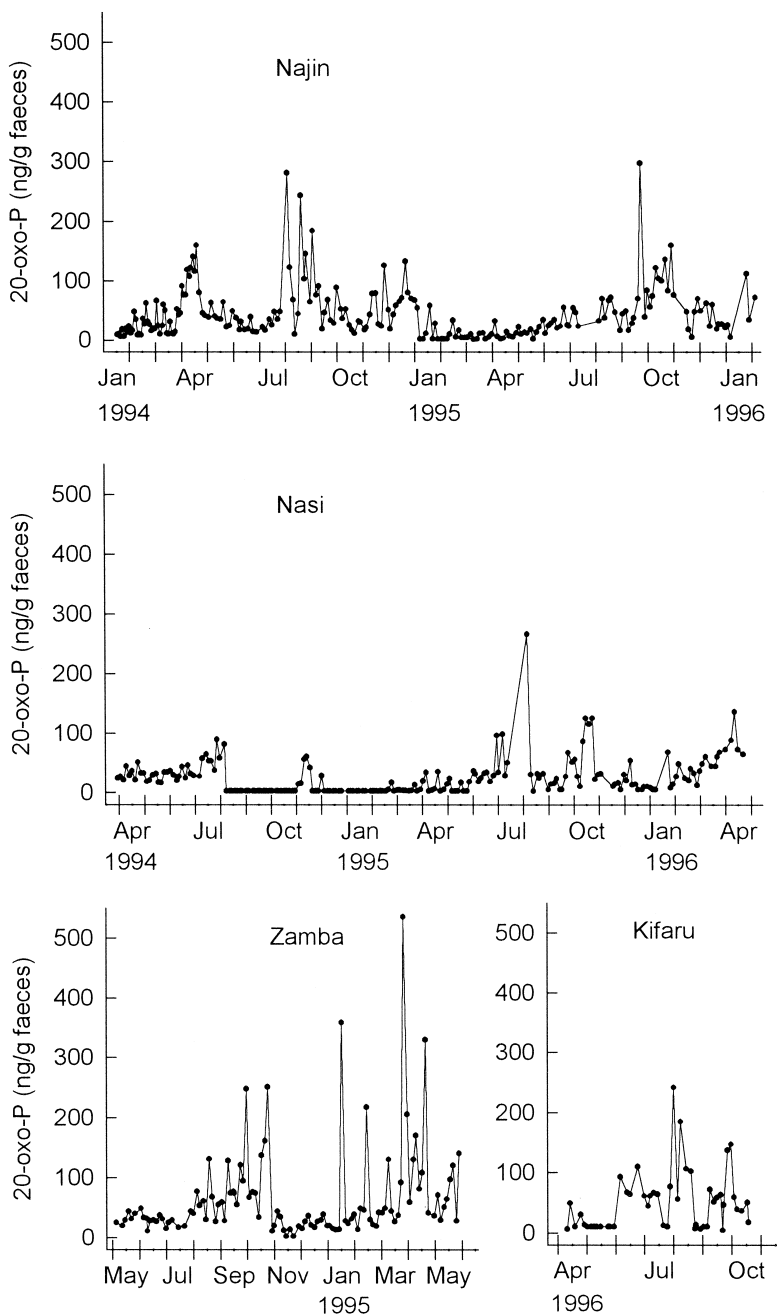


Fig. 6. Concentrations of faecal 20-oxo-P in four white rhinoceroses which, according to missing oestrous cycles (no apparent regularity), but some luteal activity indicated by 20-oxo-P values of 100–200 ng/g of faeces, were classified as category 3.

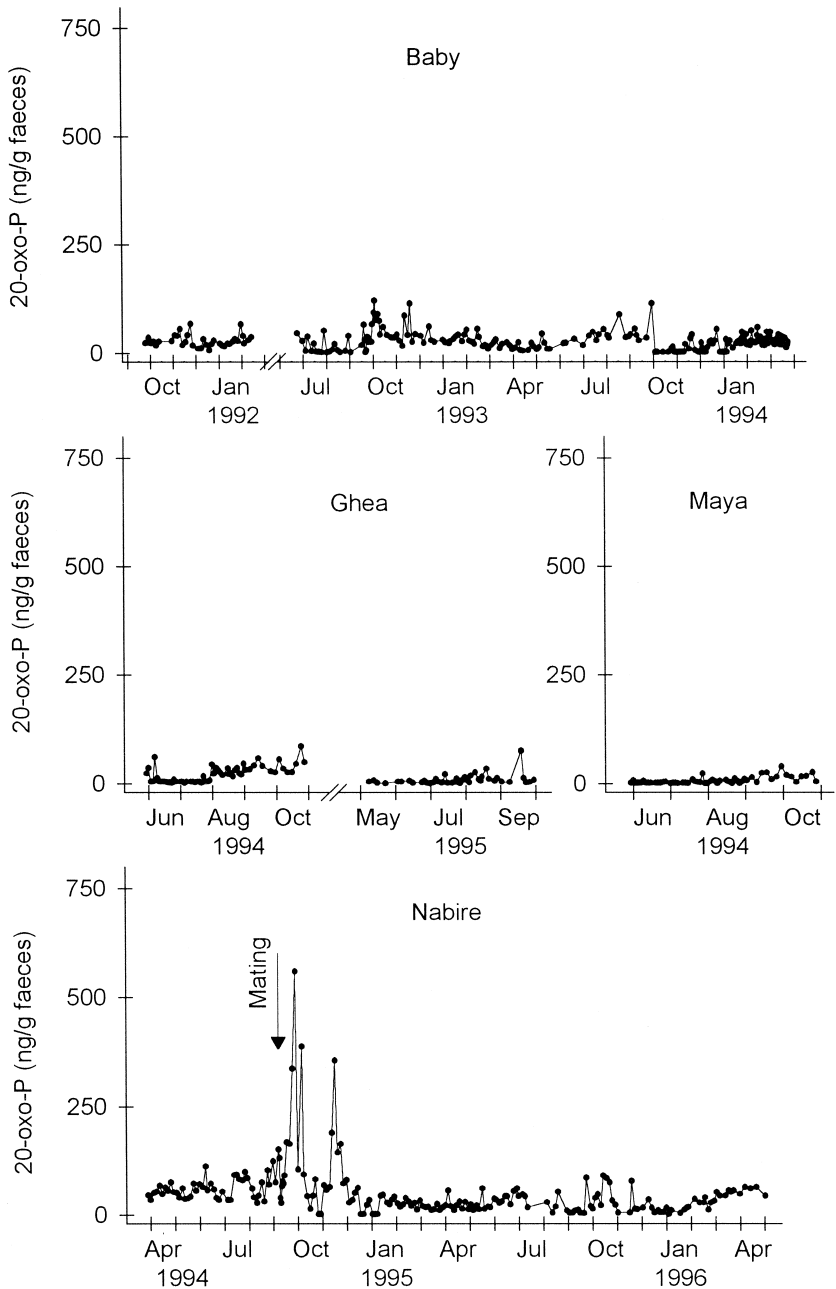


Fig. 7. Concentrations of faecal 20-oxo-P in four white rhinoceroses which, according to missing oestrous cycles and no apparent luteal activity were classified as category 4. An exception are the two luteal phases in the female 'Nabire' in 1994, which were classified as category 2.

Only two of 21 animals exhibited category 1 ovarian activity (Figs. 3 and 4), although one of those animals also exhibited category 2 activity during part of the study (Fig. 3). That female had been moved from the Munich to the Salzburg Zoo in the summer of 1991. Samples collected in October 1991 indicated an initial short, category 2 cycle followed by 10-week category 1 cycles until May 1994 (except for one shorter cycle in September/October 1993). In June, July and August 1994, this female displayed three cycles of approximately 1 month duration (30, 26 and 31 days, respectively) before re-establishing 10-week cycles. After September 1994, LP 20-oxo-P values appear to decline, and, although concentrations were at the higher end of category 2, oestrous cycle activity in this female was categorised as 2 since June 1994. The duration of category 1 cycles ( $n = 10$ ; December 1991–May 1994, except the period of February–June 1992, where no samples were available) was calculated to be  $68.5 \pm 3.5$  days; the length of the FP and LP were  $12.4 \pm 0.9$  and  $55.9 \pm 3.2$  days, respectively. The category 2 cycles (September 1994–October 1996 except January, February 1995;  $n = 11$ ) averaged  $68.9 \pm 3.3$ ,  $14.6 \pm 2.0$  and  $53.5 \pm 2.3$  days for the cycle length, the FP and the LP, respectively. Oestrus behaviour was observed primarily at the end of the LP.

The female in Fig. 4 also displayed category 1-type oestrous cycles. Because faecal samples from this animal were not collected as regularly as those from other females, an estimated cycle length of 70 days was determined by dividing the number of luteal phases ( $n = 5$ ) into the total number of days monitored (beginning of October 1994 until mid-September 1995).

Examples of category 2 oestrous cycles are shown in Fig. 5. The oestrous cycle length varied within individual animals (4–> 10 weeks) and results often differed between consecutive years in the same animal. LP and FP lengths varied between 5–> 70 ( $n = 31$ ) and 5–> 39 days ( $n = 30$ ), respectively. When the LPs was categorised as being < 35 days ( $n = 21$ ) or > 45 days ( $n = 6$ ), the mean values were

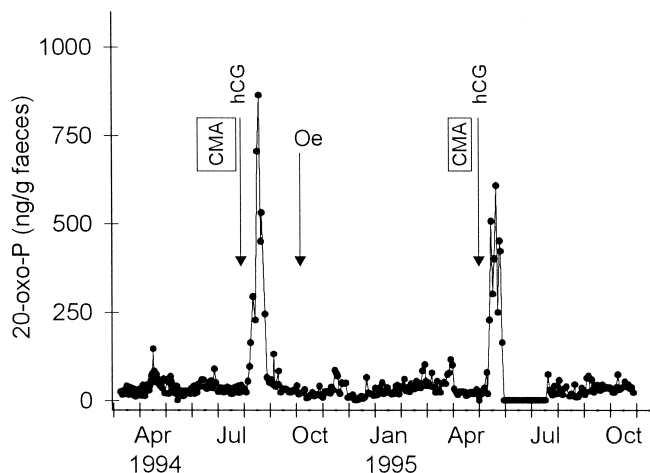


Fig. 8. Concentrations of 20-oxo-P in faecal samples of a white rhinoceros (Baby) before, during and after the treatment with the synthetic progestin chlormadinone acetate (CMA), and hCG. Oe indicates oestrus behaviour.

$16.8 \pm 2.0$  and  $58.7 \pm 4.8$  days, respectively. Mean FP length for category 2 females ( $n = 26$ ) was  $16.7 \pm 1.7$  days.

Representative category 3 animals, are shown in Fig. 6, and category 4 females are depicted in Fig. 7. The animal Nabire (Fig. 7) exhibited two category 2 luteal phases in 1994 and was mated during this period, but did not conceive. Concentration of faecal 20-oxo-P in one pregnant animal during the 4th and 5th months of gestation were  $5046 \pm 844$  ( $n = 7$ ) and  $6432 \pm 662$  ( $n = 11$ ) ng/g of faeces, respectively, and were considerably higher than those observed during the non-pregnant luteal phase.

### 3.3. Oestrous cycle induction

Faecal 20-oxo-P concentrations were low before and during each CMA treatment (Fig. 8); CMA metabolites did not affect steroid measurements. Faecal 20-oxo-P concentrations then increased approximately 10 days after hCG injection, resulting in a LP of 18 and 17 days, respectively. In response to the first treatment, 2 days of oestrous behaviour were observed 70 days after hCG injection.

## 4. Discussion

The objective of this study was to characterise long-term ovarian activity using faecal steroid analysis to try and resolve the conflicts associated with determining the oestrous cycle length in the white rhinoceros. Still, despite the continuous monitoring of some females for over 4 years, results indicate that it may be difficult to make generalisations with respect to 'normal' cycle length. Although transition between categories was flowing and some females did at certain periods fall into different groups, animals could be classified in four major categories. On the basis of regularity of interovulatory intervals (category 1 vs. category 2 animals), the 'normal' oestrous cycle in the white rhinoceros appears to be 10 weeks in length, which is considerably longer than the 25 days observed in the black (Hindle et al., 1992; Schwarzenberger et al., 1993; Berkeley et al., 1997) and Sumatran (Heistermann, 1996, personal communication) rhinoceros, and the 45 days reported for the Indian rhinoceros (Kassam and Lasley, 1981; Kasman et al., 1986). Our finding of an oestrous cycle length of 70 days contrasts with recent studies in white rhinoceroses which suggested an oestrous cycle lengths of  $\sim 1$  months (Hindle et al., 1992; Radcliffe et al., 1997). However, these studies were based on only one or two cycles per female, and there were several examples in our study where similar results of an  $\sim 1$  month cycle length would have been suggested, if we had only collected for 1–2 months.

Only two of the 21 white rhinoceroses investigated exhibited ovarian cycles characterised by high luteal phase 20-oxo-P values and regular 10-week interovulatory intervals over periods of more than 1 year. Ovarian activity in category 2 animals alternated between 4 and 10 week cycles, and LP 20-oxo-P concentrations were considerably (5–10 fold) lower than those of category 1 females. These LP concentration differences between categories are likely caused by differences in progesterone production. Sample collection site within the faecal ball did not significantly effect

results and differences in faecal water content, as well as food fibre content within physiological ranges are too minimal to explain a 5–10 fold concentration difference between categories. There was also no age effect with respect to categorising of ovarian activity and season also was not a factor because at least two females cycled year round.

Defining the 70 day cycles in category 1 animals as resulting from persistent corpora lutea is not an adequate explanation because these interovulatory intervals were too regular. Persistent corpus luteum activity in mares without uterine anomalies or infections is characterised by highly variable LP lengths ranging between 35 and 95 days (Stabenfeldt et al., 1974). Conversely, the presence of an inflammatory uterine infection generally reduces progesterone concentrations during dioestrous and causes early regression of the corpus luteum (Ginther, 1992). Evidence of intrauterine fluid accumulation has been identified using ultrasound twice during early embryonic resorption in one 33-year old white rhinoceros (Radcliffe et al., 1997). The two conceptions observed by these authors were characterised by interovulatory intervals of  $\sim 11$ –12 weeks. Early embryonic death in both pregnancies occurred 28 days post ovulation, but faecal progestagens were elevated for  $\sim 44$  days. This study demonstrate that category 2 animals can conceive, whereas the fertility potential of category 1 females remains to be determined.

In the study of Radcliffe et al. (1997) the first 3 months of evaluation were characterised by short luteal phases with low faecal progestagen concentrations and no ovulations. Instead, the ovary contained a large follicle with luminal fibrous bands, which disappeared gradually analogous to an equine haemorrhagic follicle. In another study, a persistent follicular cyst-like structure was monitored by rectal ultrasonography over a 34 day observation period in one white rhinoceros (Adams et al., 1991). Again, no ovulation occurred and no corpus luteum was observed. These findings suggest that category 3 and 4 animals could have cystic structures that may or may not become luteinized. Results of these and the present studies also suggest that anovulation may be a major problem in the captive white rhinoceros population. Animals in categories 1 and 2 were apparently the only animals in which ovulation occurred regularly, whereas nearly two-thirds of the remaining animals (category 3 and 4 animals) did not ovulate.

Although our treatment regimen using chlormadinone acetate followed by an hCG injection failed to induce continuous cyclicity, results indicate that ovulation and subsequent corpus luteum activity can be induced in white rhinoceroses. The LP 20-oxo-P concentrations increased  $\sim 10$  days after hCG injection. These findings corroborate the ultrasound findings during natural ovulation, which was detected 7–9 days before a substantial rise in faecal progestagens (Radcliffe et al., 1997). Because ovulation of a preovulatory follicle usually occurs within 48 h after hCG injection (Ginther, 1992), these two days plus the 7–9 days for the development of a steroidogenically functional corpus luteum add up to the 10 days observed in our study. Also comparable to Radcliffe et al. (1997) mounting by a bull or mating in several occasions in our study occurred  $\sim 10$  days before the next LP.

Elution profiles of immunoreactive 20-oxo-P were comparable to those of pregnant black rhinoceroses, excluding a small peak coeluting with [ $^3$ H]progesterone (Schwarzenberger et al., 1996b). The finding of different immunoreactive 5 $\alpha$ -pregnanes agrees with studies on faecal metabolites in several other mammalian species (for review see

Schwarzenberger et al., 1996a, 1997), but contrasts with a study by Hindle and Hodges (1990) who administered radiolabelled progesterone to a white rhinoceros and found only one radioactive peak of in the faeces which coeluted with progesterone after HPLC-separation. In contrast to faecal samples in black rhinoceroses, where pregnanes containing a 20-oxo-group or a 20 $\alpha$ -OH-group seem to be equally important, faecal pregnanes containing a 20-oxo-group dominate over those containing a 20 $\alpha$ -OH-group in the white rhinoceros (Schwarzenberger, unpublished observations). This finding is also supported by concentration differences in faecal samples of both species. Luteal phase 20-oxo-P concentrations in faeces of the white rhinoceros exceed 1000 ng/g, compared to  $\sim$  500 ng/g in faeces of the black rhinoceros (Schwarzenberger et al., 1993, 1996b).

Our preliminary results of the faecal progestagen concentration in the one pregnant white rhinoceros suggested that foetoplacental progesterone metabolite production can be used for pregnancy diagnosis as in other rhinoceros species (Kasman et al., 1986; Ramsay et al., 1987; Hodges and Green, 1989; Kuckelkorn and Dathe, 1990; Kock et al., 1991; Schwarzenberger et al., 1993, 1996b; Ramsay et al., 1994; Czekala and Callison, 1996; Berkeley et al., 1997). Gestation length in the white rhinoceros is  $\sim$  15 months and the faecal 20-oxo-P values during the 4th and 5th month of pregnancy were already considerably higher than luteal phase concentrations. Hodges and Green (1989) also reported high urinary pregnanediol concentrations during the second half of gestation in a white rhinoceros.

In conclusion, on the basis of differences in oestrous cycle length and LP 20-oxo-P concentrations among animals, white rhinoceroses could be classified into four categories. Animals in category 1 and 2 appeared to ovulate, resulting in either regular oestrous cycles of 10 weeks in duration ( $n = 2$ ) or variable cycles of 4–10 weeks in length ( $n = 6$ ). In contrast, faecal 20-oxo-P values in almost two thirds ( $n = 13$ ) of the white rhinoceroses in this study indicated erratic or missing luteal activity, apparently attributable to anovulation.

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