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Non-invasive hormone analysis for reproductive monitoring in female southern white rhinoceros (*Ceratotherium simum simum*)

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Abstract
*The reproductive status of captive female southern white rhinoceros (*Ceratotherium simum simum*) was monitored non-invasively using an enzyme-linked immunosorbent assay measuring faecal 4-pregnen-20α-ol-3one (20α-hydroxyprogesterone, 20α-OHP). Matched faecal and plasma or urine samples were collected two to three times per week from eight female animals, housed at three zoological establishments in the UK for six months. The faecal samples were dried, crushed and the steroid hormones extracted with potassium hydroxide and methanol prior to analysis. The data were contrasted with urine or plasma steroid concentrations determined using previously validated assays (Hindle et al., 1992), for confirmation of our findings. Using this method we identified two pregnant, five cyclic and one acyclic animal. In cyclic females, the mean (±SD) faecal immunoreactive 20α-hydroxyprogesterone concentrations varied between 501.2 ± 158.1 ng/g dry faeces during the luteal period and 158.1 ±14.7 ng/g dry faeces in the inter-luteal period, which corresponded with the period when matings were observed. The acyclic female showed no comparable cyclic variation in hormone excretion; sample concentrations remained around 177.3 ± 97.5 ng/g dry faeces. In the pregnant animals, concentrations of faecal progestagens rose significantly above mean luteal levels after the first fifty days of gestation, and remained elevated at an average of 3637.5 ±1192 ng/g dry faeces until the end of the study. One female has produced a live calf 15 months after the predicted conception date. The mean oestrous cycle length was 32 ± 1.85 days, with mean inter-luteal and luteal periods of 7.6 ±1.3 and 25.6 ±1.6 days respectively. These data demonstrate that under appropriate management conditions, this technique provides a valuable asset for reproductive monitoring and herd management in the southern white rhinoceros.*

Introduction

Wild female southern white rhinoceros reproduce regularly, with average inter-calving intervals of 2.5 years (Owen-Smith, 1975). However, reduced reproductive success has been reported in captivity (Patton *et al.*, 1999), and is reflected in the UK population of this species. Of the five extant rhinoceros species, the southern white is currently the least endangered (Foose, 1999), numbering some 8400 free-living animals, and 700 animals in captivity (Göltenboth & Ochs, 1997), but its poor reproductive performance in captivity and the ever-present pressure of poaching on wild populations result in its survival remaining conservation dependant.

Both urinary and faecal analyses have been used to study reproductive events in white rhinoceros (Hindle *et al*., 1992; Radcliffe *et al*., 1997; Schwarzenberger *et al.*, 1998; Patton *et al.*, 1999), but the data available is often conflicting and the hormonal profile of the reproductive cycle and pregnancy has yet to be fully characterised. A number of practical issues contribute to this problem: sample collection for non-invasive endocrine analysis is difficult in group-housed animals; many of the reproductively active females are pregnant most of the time and hence rarely exhibit non-conceptive cycles; the remaining animals available for study are often non-reproductive and exhibit erratic cycles or none at all (Schwarzenberger *et al.*, 1998). The current study aimed to develop a practical method for monitoring reproduction in this species, using faecal hormone metabolite analysis and which was validated through comparison of the data with results obtained from matched urine or plasma samples.

Materials and methods

Animals and study sites

Eight animals of unknown reproductive status from three different zoological collections within the UK were studied (table 1). Diets were consistent between the different groups, although the Paignton Zoo (PZ) animal, had no free access to natural graze during the winter. Approximately 20g – 50g of faeces per animal was collected fresh, two or three times per week, and stored frozen at –20ºC. For the Whipsnade Wild Animal Park (WWAP) animals, venous blood was collected in 20ml heparinised evacuated tubes (Vacutainer, Becton Dickinson Vacutainer Systems, Plymouth, UK) from the dorsal ear vein on the same day as the faecal samples. No restraint was used. Blood samples were centrifuged for two minutes and the plasma separated and stored frozen at –20ºC in 2ml plastic vials. For the remaining individuals either free-flow or floor-drainage urine samples were collected and 10 to 15 ml were stored frozen at –20ºC in plastic universal containers.

Name	*Sex*	*Provenance*	*Age In Years*	*Location*	*Reproductive category after sample analysis*
Trio	Female	Captive born	23	WWAP	Pregnant
Mikumi	Female	Captive born	9	WWAP	Pregnant
Clara	Female	Captive born	19	WWAP	Cyclic
Toots	Female	Wild born	7	WMSLP	Cyclic
Mtuba	Female	Wild born	9	WMSLP	Cyclic
Trixie	Female	Wild born	5	WMSLP	Cyclic
Zulu	Female	Wild born	9	WMSLP	Acyclic
Gracie	Female	Captive born	20	PZ	Cyclic

WWAP: Whipsnade Wild Animal Park, Dunstable, Bedforshire, England.
WMSLP: West Midlands Safari and Leisure Park, Bewdley, Worcestershire, England.
PZ: Paignton Zoological and Botanical Gardens, Paignton, Devon, England.

Table 1. Summary of the southern white rhinoceroses used in this study and their reproductive status as determined from the results of the analysis

Assay procedures

Faecal and urine samples were analysed for their 20α-OHP content using the assay procedure previously described by Hindle *et al.* (1992). The sensitivity of the assay was 0.13 ng/ml as determined at 80% B/Bo. The interassay coefficient of variance (CV) was 27.4% (N = 16) for the high (6.4 ng) quality control (QC), 6.4% (N = 16) for the low (0.1 ng) QC and 18.03% (N = 16) for a 1.3 ng QC. Plasma progesterone concentrations was measured by radioimmunoassay (RIA) using a method similar to that described by Shaw *et al.* (1989).

Urine sample steroid concentrations were indexed to their creatinine content (Hodges & Green, 1989) to account for variations in fluid volume. Prior to analysis by 20α-OHP ELISA, the samples were hydrolysed and extracted as described by Hodges *et al.* (1979). Concentrations of urinary 20α-OHP are, therefore, expressed in ng/mg creatinine.

Faecal samples were thawed and dried in a laboratory oven for 18 h at 40°C prior to extraction. The entire sample was then thoroughly mixed, pulverised and sieved through a 1.0 by 1.5mm sieve to separate the faecal powder from the coarse undigested hay. Several potential extraction procedures were evaluated and the optimum method found to be that described by Shaw *et al.* (1995). The extracted faecal material was diluted 1:40 with assay buffer for non-pregnant animals and 1:200 for pregnant animals before analysis. Sample concentrations are expressed as ng/g dried faecal powder.

Data analysis

Faecal hormone concentrations were contrasted with corresponding urine or plasma data. Visual interpretation of the faecal 20α-OHP data suggested inter-luteal phase concentrations <200 ng/g faeces. Based upon this observation, and the work of Plotka *et al.* (1988), Schwarzenberger *et al.* (1998) and Patton *et al.* (1999), the inter-luteal phase was defined as a period of eight to ten days during which a minimum of three samples <200 ng/g faeces could be demonstrated. In cases where sampling was less than three times a week, speculative assumptions were made that these periods equated to inter-luteal phases. The onset of the luteal phase was considered as the first of two consecutive samples of successively increasing concentration, which exceeded the mean + 2SD of the preceding inter-luteal phase. The last inter-luteal phase sample and first luteal phase samples were all collected within a seven-day period. Separate mean ± standard deviation (SD) inter-luteal and luteal phase concentrations were calculated for each cycle. In the same way, a baseline of 2ng/mg creatinine was assumed for urine analysis.

The duration of the oestrous cycle was calculated as the time between the onset of two successive luteal phases. Cycle length was calculated separately for each individual for the faecal, plasma or urinary values, as applicable.

Results

Matched plasma progesterone and faecal 20α-OHP concentrations showed a significant correlation (Pearson's product moment correlation coefficient $r^2 = 0.36$, $p < 0.0001$, $N = 38$). The correlation between urinary and faecal 20α-OHP concentrations was not as strong ($r^2 = 0.24$) but still significant ($p < 0.0001$, $N = 59$). Faecal 20α-OHP analysis suggested that two of the eight females sampled were pregnant, five were cyclic and one was acyclic animal.

In the presumed pregnant animals, during the first fifty days of gestation, the mean faecal 20α-OHP concentration was 408.7 ± 45.5 ng/g and not significantly different from the mean luteal concentration in non-pregnant animals (t-test: two-sample assuming unequal variances, $p > 0.1$, $N = 2$, $N = 5$). However, after the first fifty days of pregnancy, the faecal concentrations rose to a mean of 3637.5 ± 1192 ng/g, significantly different to the non-pregnant luteal concentration mean (t-test: two-samples assuming unequal variances, $p < 0.05$, $N = 2$, $N = 5$). For one pregnant female, Trio, plasma progesterone concentrations rose almost immediately after presumed conception (retrospectively confirmed by parturition occurring 15 months later) and frequently exceeded the upper limit of detection of the assay (>6.0ng/ml, fig 1a.). For the other female (Mikumi; fig 1b.), plasma concentrations showed no significant increase and tended to be similar to those observed in the non-pregnant female which exhibited oestrous cycles (Clara; pregnant mean = 2.31±0.48ng/ml vs 2.18±1.61ng/ml during oestrous). Nevertheless, the diagnosis of pregnancy based on the characteristics of the faecal profile was confirmed by parturition 15 months after the presumed date of conception.

In cyclic animals, the average oestrus cycle length was 32 ± 1.85 days (based on seven complete cycles in five females) with average luteal phases of 25.6 ±1.6 days and inter-luteal periods of 7.6 ± 1.35 days. The mean faecal 20α-OHP concentrations were 158.06 ± 14.7 ng/g during the inter-luteal phase and 501.2 ± 158.1 ng/g during the luteal phase (fig. 2). Urinary 20α-OHP concentrations ranged from 0.018 to 28 ng/mg creatinine, with a mean of 5.2 ± 5.6 ng/mg creatinine. Urinary 20α-OHP profiles did not reveal as many complete oestrous cycles as the faecal profiles. Cycles detected were generally shorter than those determined using faecal profiles (25±1.1 days) with 16 day inter-luteal and 9 to 10 day luteal intervals. The acyclic female had low faecal and urinary 20α-OHP concentrations which showed no cyclic variation.

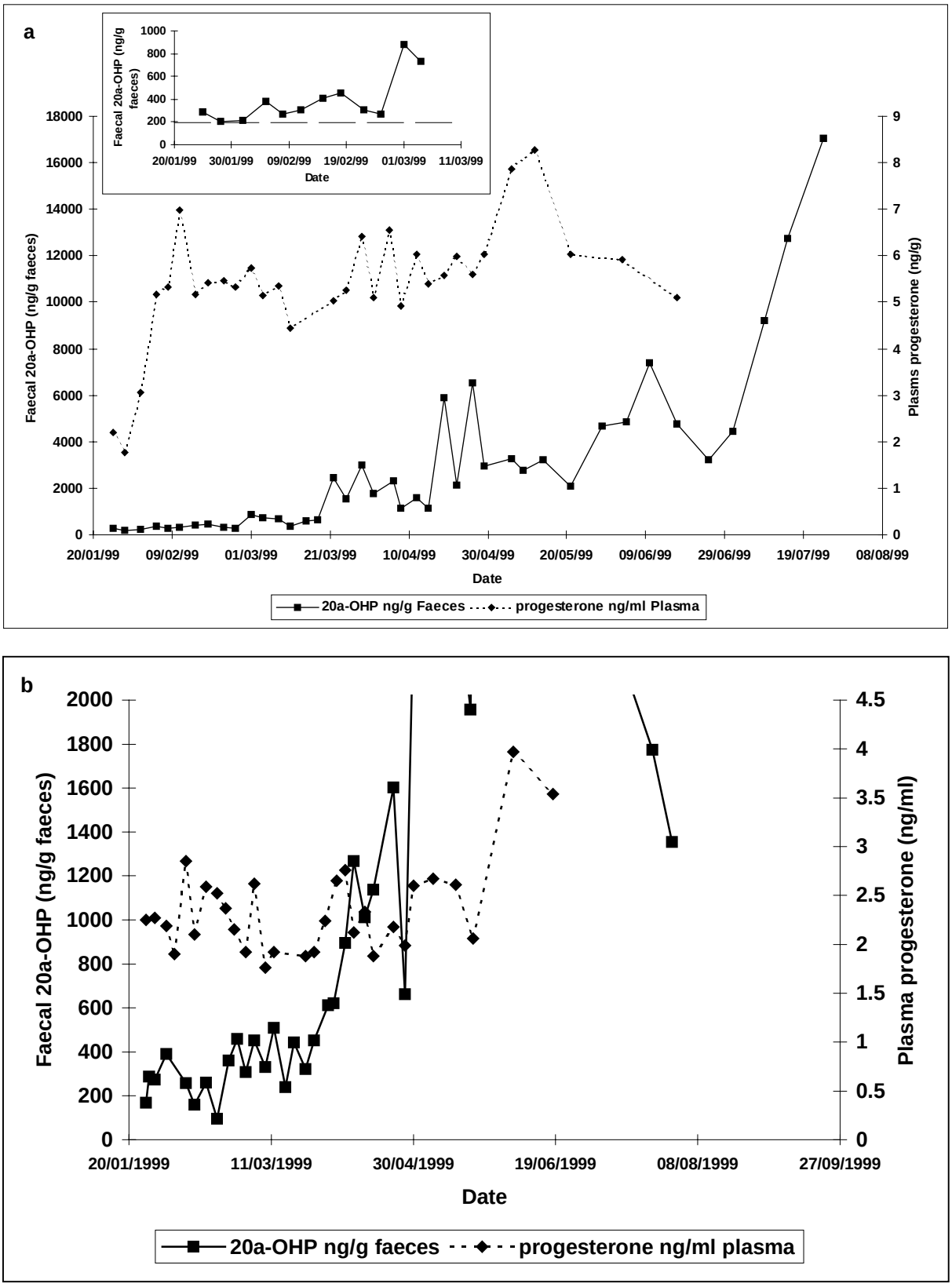

Figure 1. a) Faecal and plasma progestagen concentration in pregnant white rhino "Trio", inset shows the faecal hormone concentrations over a larger scale. b) Comparable profile for pregnant female Mikumi.

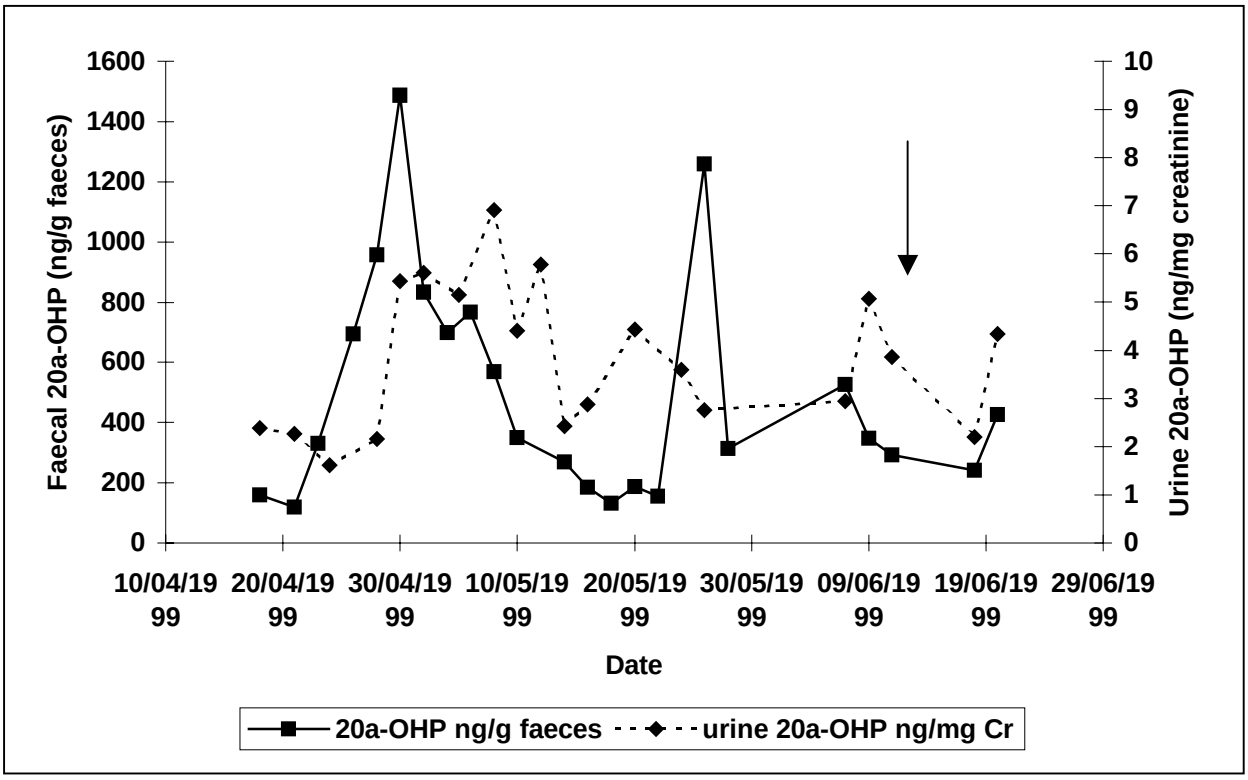

Figure 2. A typical profile from a cyclic female (Toots) showing faecal and urinary hormone concentrations. The arrow indicates observed mating behaviour.

Discussion

Faecal progestagen profiles were used to accurately diagnose pregnancy in two females. From the fiftieth day of gestation both animals showed a rise in faecal progestagen concentrations to levels significantly higher than average luteal concentrations. This finding corresponds with the results of Patton *et al.*, (1999), who noted a rise in faecal progestagen levels between the first and third month of gestation. Levels continued to increase until the seventh month of gestation before remaining at a relatively constant high level until the end of the study period. These observations suggest that pregnancy can be diagnosed in the southern white rhinoceros as early as two months post-conception, but regular, frequent sample collection is vital for accurately interpreting the changes in the longitudinal hormone profile and predicting parturition dates with any degree of accuracy.

Significant differences in the plasma progesterone profiles of the two pregnant females existed during the early stages of gestation, which would have resulted in differential diagnoses in the absence of the faecal data. Trio's plasma progesterone concentration was low for the first three samples collected, presumably around the time of ovulation and conception (categorised as an inter-luteal phase from the faecal data). Plasma concentrations then rose precipitously and remained elevated until the end of sampling. Trio gave birth to a female calf on 1st May 2000, 15 months exactly after her presumed conception at the end of January 1999. Mikumi's plasma progesterone levels were within the normal cyclic range until 70 days after the presumed date of conception, after which they doubled in concentration. The lack of obvious cycles in the faecal data led us to presume that conception had occurred around 21st February 1999, when faecal hormone concentrations were fluctuating about the 200ng/g level, indicative of the inter-luteal phase. This female gave birth on the 19th May 2000, once again 15 months after the presumed date of conception. Both gestations were within the normal range recorded for this species.

Previous studies (Radcliffe *et al.,* 1997; Schwarzenberger *et al.,* 1998; Patton *et al.,* 1999) have suggested the frequent occurrence of extended luteal phases in captive white rhinoceros females, which may be a factor involved in the reduced reproductive rates of this species. One of the five cyclic females studied (Clara) exhibited two periods of elevated progestagen concentrations lasting 49 and 60 days. However, she also exhibited temporal changes which fitted the criteria for a presumed oestrous cycle of approximately 35 days duration. It remains to be determined whether the extended periods of elevated progestagens were due to infrequent sample collection resulting in an absence of inter-luteal samples, or truly aberrant cycles. Aberrant cycles may be caused by pathological conditions such as endometritis or pyometra, but further clinical investigation would be required to confirm this.

Zulu demonstrated erratic, non-cyclical progestagen concentrations persistently below the average luteal concentrations in all of the cyclic animals and has not shown signs of behavioural oestrus for over six months. This is a common observation for captive southern white rhinoceroses (Schwarzenberger *et a.l,* 1998; Patton *et al.,* 1999) and further monitoring would be needed to establish whether she remains acyclic or exhibits periods of normal cyclic activity.

Our findings suggested that differentiation between normal and aberrant cyclic activity was not possible when less than two regularly spaced samples were collected per week. Such intensive monitoring may interfere with management, especially in extensive herd situations, but would be essential for assessing females with poor reproductive records. For early pregnancy diagnosis, the collection of two to three samples per week over an initial three month period is required. Thereafter, pregnancy may be monitored by weekly sampling. Faecal sampling may require the isolation of an animal until it has defecated, or close observation of the animals to ensure sample identification in a herd situation. Nevertheless, it remains a simpler procedure than either urine or plasma collection, and these data suggest that faecal analysis may provide more practical information than either urine or plasma analysis.

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