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Non-invasive diagnosis of pregnancy in wild black rhinoceros (*Diceros bicornis minor*) by faecal steroid analysis

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Abstract. The objective of this study was to determine whether faecal progestagen measurement could be used to diagnose pregnancy in wild black rhinoceros cows. Immunoreactive 20α-progestagens were measured in faecal samples collected regularly (one or two times times per week) from pregnant and non-pregnant wild black rhinoceros females (n = 6) in Zimbabwe. Fresh dung piles deposited by the study animals were serially sampled during prolonged periods of tracking with local game scouts. Samples were stored frozen, and dried prior to methanol extraction. Immunoreactivity in faecal extracts was measured with a 20α-dihydroprogesterone enzyme immunoassay and was shown to reflect circulating progesterone concentrations. Mean concentrations of faecal 20α-progestagens during each month of gestation were significantly higher than faecal concentrations in non-pregnant animals (P<0.05), except during the second month of gestation. Faecal 20α-progestagens remained 5–10 times higher than concentrations in non-pregnant animals from the 4th to 15th month of gestation. It was concluded that regular non-invasive reproductive monitoring of black rhinoceros in the wild was possible and that pregnancy could be accurately diagnosed from the measurement of 20α-progestagens in faecal samples. The use of this technique in wild black rhinoceros populations will offer new perspectives for *in situ* management of this endangered species.

Extra keywords: 20α-dihydroprogesterone enzyme immunoassay, reproductive success.

Introduction

The black rhinoceros in the wild has suffered one of the most dramatic declines in Africa. The species, which once occupied most of sub-Saharan Africa and numbered in the hundreds of thousands, has been decimated through poaching for its horn (Western and Vigne 1984). In order to reduce losses due to poaching, a number of management operations have been undertaken *in situ*, including the increased protection of remaining animals and the establishment of breeding nuclei in safer areas. The black rhinoceros population has now stabilized at approximately 2400 animals (Brooks 1996), but only one single unfenced population with more than 100 individuals remains (Berger 1994). The conservation priority for such small and fragmented populations is now to rebuild numbers as quickly as possible in order to reduce the genetic and demographic risks of extinction (Emslie 1994). Prerequisites for this are to ensure that each female has maximum breeding output, and subsequently to have the ability to assess the reproductive success in the animals.

Traditionally, the diagnosis of pregnancy in wild animals in their natural environment has been established retrospectively by observations after birth. However, black rhinoceros are characterized by long life parameters, with a gestation of 15 months (Goddard 1967; Hall-Martin and Penzhorn 1977) and calving intervals ranging from 2 to 5 years in the wild (Goddard 1967; Joubert and Eloff 1971; Hitchins and Anderson 1983; Hall-Martin and Penzhorn 1977; Adcock 1996). This means that the retrospective evaluation of reproductive output in wild black rhinoceros necessitates a minimum of 5 years of observation. In addition, birth rates are difficult to evaluate accurately in wild black rhinoceros because the detection of a newborn calf can be extremely arduous under natural conditions (Garnier, unpublished data). Such a method is inappropriate for the effective management of black rhinoceros breeding nuclei, which requires immediate, accurate and repeatable assessment of the reproductive status of individual animals.

Invasive procedures, such as the measurement of serum progesterone or transrectal ultrasound examination, have been successfully used to monitor gestation in captive African rhinoceros (Radcliffe *et al.* 1996, 1997; Berkeley *et al.* 1997) and wild black rhinoceros (Kock *et al.*, 1991), but these techniques necessitate the chemical or physical restraint of the animals. Non-invasive urinary steroid analysis has been used in captivity for monitoring ovarian events in both African (Ramsay *et al.* 1987; Hindle *et al.* 1992) and Indian rhinoceroses (Kassam and Lasley 1981; Hodges and Green 1989). However, when Brett *et al.* (1989) tried to use this technique in the wild, the frequent collection of repeated

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urine samples from black rhinoceros proved too difficult. The measurement of progesterone metabolites in saliva has allowed for pregnancy detection in captive black rhinoceros (Czekala and Callison 1996; Thorne *et al.* 1998). Because collection of faecal samples is far more practical, faecal steroid measurement is now used for reproductive assessment in an increasing number of wildlife species in captivity (see Schwarzenberger *et al.* 1996*a* for review), including white rhinoceros (Radcliffe *et al.* 1997; Schwarzenberger *et al.* 1998) and black rhinoceros (Schwarzenberger *et al.* 1993, 1996*b*; Berkeley *et al.* 1997). Oestrous cycles have also been characterized in the Sumatran rhinoceros by the use of both urinary and faecal steroid analysis (Heistermann *et al.* 1998).

In the wild, faecal steroid analysis has been used in fewer species. In free-roaming feral horses and bisons, faecal steroid metabolites proved to be accurate predictors of pregnancy and also provided an assessment of corpus luteum activity (Kirkpatrick *et al.* 1990, 1991, 1992, 1993). Using this technique, ovarian cycles were also characterized in wild cotton-top tamarins, baboons and muriqui monkeys (Wasser 1996; Savage *et al.* 1997; Strier and Ziegler 1997). Pregnancy could also be detected in caribou (Messier *et al.* 1990) and in elk (White *et al.* 1995), and reproductive patterns were studied in African wild dogs (Creel *et al.* 1997). In free-ranging males, urinary steroid analysis was used to measure reproductive hormones in African elephants (Poole *et al.* 1984) and mountain gorillas (Robbins and Czekala 1997).

The main objective of our research was, therefore, to investigate the potential use of faecal progestagen measurement for pregnancy diagnosis in wild black rhinoceros females. In particular, we wished to (*1*) determine whether faecal progestagen concentrations reflected serum progesterone concentrations in wild females, (*2*) measure faecal progestagen concentrations during pregnancy, and (*3*) establish whether faecal progestagen measurement could be used as a reliable indicator of pregnancy in wild black rhinoceros females.

Materials and methods

Animals

Six female black rhinoceros of the southern subspecies (*Diceros bicornis minor*), including five wild and one semi-wild animals, were studied in Zimbabwe. Both wild and semi-wild situations are characterized by a natural breeding system, but differ in the size of the area and in the animal density, as well as in the availability of supplementary food and the management intensity (Bride *et al.* 1996).

Five wild females were studied in the Save Valley Conservancy (20E, 31S), which had a population of 60 black rhinoceros in July 1998. The Conservancy has a surface area of 3387 km^2 and a vegetation cover consisting mainly of *Colophospermum mopane* woodland, *Acacia-Combretum* woodland-savannah and riverine vegetation (Du Toit and Price Waterhouse 1994). Four females were translocated to the Save Valley in 1986–88 and were estimated to be between 25 and 37 years of age when the parturition recorded in this study occurred. Age estimation was based on toothwear pattern (Du Toit 1986). One female was the offspring of a translocated animal and was 9 years of age at the time of parturition during this study. The decision to study these females was based on their presence in the same area in the Conservancy and on the fact that they were multiparous. Their last calving intervals ranged from 23 to 38 months.

The semi-wild female was studied at Imire game ranch (18E, 31S), to which three male and four female black rhinoceros were translocated as orphans in 1987, at between 2 and 5 months of age. The animals were hand-reared and thereafter fed on naturally occurring browse in the area, where the main vegetation types are *Brachystegia* and *Julbernardia* woodland and open grassland. Their food is also supplemented daily with game cubes. All seven animals are kept in an enclosure at night and let out to feed in the bush during the day. The semi-wild female was nearly 11 years old when she gave birth to her first calf during the study. Wild and semi-wild animals are hereafter referred to as 'wild'.

Data collection

In the Save Valley Conservancy, two females were monitored throughout the entire gestation and the three others were monitored during the last 9 months of gestation. Sample collection occurred once or twice per week during the first and last trimester of pregnancy and every week during mid-pregnancy. Sample collection frequency was initially planned using knowledge of previous calving intervals. Frequency was then adjusted in line with environmental conditions and was also governed by the on-going results of hormonal analysis. Samples from non-pregnant animals were collected two or three times per week, during the 3 months before the onset of pregnancy ($n = 2$) or between 4 and 7 months following parturition ($n = 3$), because some black rhinoceros females in the Save Conservancy conceived as soon as 8 months after parturition.

The animals were identified and located by the use of radio-tracking, in the case of one radio-collared female, and by tracking with local game scouts for the other females that were not collared. Finding, identifying and tracking an individual rhinoceros consisted of searching for its characteristic footprint at dawn around water holes within her home range. Each individual female was characterized by the size and markings of her footprint, as well as by the features of her calf's footprint. Characteristic footprint markings included the width between the two side toes and the pattern of wrinkles in the base of the footpad (Brett *et al.* 1989). The animal's trail or radio-signal was followed until her freshest dung pile could be found. A dung pile was considered to be fresh when the superficial layer of faecal pellets was still wet and no insect contamination had occurred. Footprint identification near the dung pile confirmed that the excreta originated from the study animal. Faecal samples were collected by breaking apart a faecal pellet and pushing its contents into polythene bags using a wooden stick. Samples were placed in a thermos flask at ambient temperature, for a period ranging from 30 min to 4 h, until they could be stored in an electric cool box. They were then transferred to permanent freezing facilities and stored at –18°C. When conditions were favourable so that the animal would remain undisturbed (animal upwind, vegetation not too dense), tracking continued until the female was sighted. Any interactions between this female and males in the area were also recorded, either directly when sighting the animals or indirectly by observing footprints while tracking.

In the Imire Game ranch, faecal samples were collected off the ground every 3–5 days for 2 months before pregnancy, and for the first 6 months and the last 2 months of pregnancy. Between the 8th and 13th month of pregnancy, one sample was collected every 5–10 days. The occurrence of mating and oestrous behaviour was recorded daily.

Paired serum and faecal samples were also collected from 15 wild females, including heifers and multiparous females, which were immobilized during management operations in the Save Valley Conservancy and Hwange National Park in Zimbabwe in 1994 and 1995. The reproductive status of these females was established retrospectively after observation of the birth of a calf.

Before being shipped to London every 8–9 months, samples collected from wild animals were thawed and dried at 65°C for 18 h in an oven (Labotec, Johannesburg, South Africa). They were subsequently sifted to remove coarse vegetation debris and stored at 4°C until extraction.

Gestation length was measured as the interval between the last observed mating and parturition. When no mating was observed, it was determined on the basis of faecal hormonal measurement in relation to the observations of interactions with a male.

Faecal sample extraction and analysis by enzyme immunoassay

Methanol extraction was used to remove steroids from solid faecal matter, by combining 0.1 g dry faeces with 0.2 g aluminium oxide, 0.6 mL methanol and 0.5 mL distilled water (Möstl *et al.* 1993). Aluminium oxide was used to reduce colorimetric interference when quantifying the extent of the reaction (Schwarzenberger *et al.* 1996*b*). The suspension was vortexed for 10 min and centrifuged at 1720*g* for 30 min at 4°C. The supernatant was diluted with 0.02 M Tris buffer (pH 7.5), 1 : 50 for non-pregnant animals and 1 : 100 or : 200 for pregnant females.

Faecal progestagens were measured using the enzyme immunoassay (EIA) validated and described by Hindle *et al.* (1992) for measuring 4-pregnen-20α-ol-3-one (20α-dihydroprogesterone, 20α-OHP) in African rhinoceros urine. The EIAs were performed in microtitre plates coated with an antibody against IgG, using a double antibody technique. The antibody was raised in rabbits immunized against 20α-OHP conjugated to bovine serum albumin through carboxymethyloxime (donated by Dr M. J. Peddie, Department of Physics and Pharmacy, University of Southampton). The antiserum showed cross-reactivity with 5β-pregnan-20α-ol-3-one (47.2%), 5α-pregnan-3β,20α-diol (11.1%), 5α-pregnan-3α,20α-diol (1.4%), 5α-pregnan-3,20-dione (0.31%), 5β-pregnan-3,20-dione (0.26%), 5β-pregnan-3α,20α-diol (0.15%), 5β-pregnan-3β,20α-diol (0.11%), 5β-pregnan-3β-ol-20-one (0.11%), 5α-pregnan-3α-ol-20-one (0.1%), 5α-pregnan-3β-ol-20-one (0.05%), and 5β-pregnan-3α-ol-20-one (0.013%). The enzyme label was biotin conjugated to 20α-OHP (donated by Dr E. Möstl, Institut für Biochemie, Veterinärmedizinische Universität, Vienna, Austria). Antibody and enzyme label dilutions were 1 : 150 000 and 1 : 800 000 respectively.

The standard curve was prepared with 20α-OHP (Sigma P6288, Sigma Chemical Co., Poole, Dorset, UK) and ranged from 0.003125 to 0.8 ng per well. Serial dilutions of faecal extracts gave a displacement curve parallel to that obtained with 20α-OHP standards. The accuracy of the assay was determined by adding different amounts (range, 0.025–0.2 ng per well) of unlabelled 20α-OHP to faecal samples containing low concentrations of endogenous hormone. Recovery of exogenous 20α-OHP was 87.9 ± 4.2%. The average sensitivity of the assay, determined as 90% binding, was 0.0625 ng per well. The intra-assay coefficients of variation were 17.7% and 5.2% for high (3.37 ± 0.51 ng mL^{-1}) and low (0.28 ± 0.10 ng mL^{-1}) concentration quality control samples respectively, and the inter-assay coefficient of variation was 18.4%.

Because the antiserum cross-reacted with 20α-hydroxylated progestagens other than 20α-dihydroprogesterone, immunoreactivity measured in faecal extracts will be referred to as immunoreactive 20α-progestagens. Hormone data were expressed as ng g^{-1} of faeces (dry weight).

Serum samples extraction and analysis by radio-immunoassay

Blood samples were collected via venipuncture in the ear during management procedures. The serum was recovered and stored at –20°C until analysis.

Progesterone was measured in serum samples by radio-immunoassay (RIA), as described by Shaw *et al.* (1989), which used a sheep anti-progesterone antibody cross-reacting with 11α-hydroxy-4-pregnen-3,20-dione (29.8%), 11β-hydroxy-4-pregnen-3,20-dione (16.5%), 5β-pregnan-3α,20α-dione (16.1%) and 5α-pregnan-3α,20α-dione (2.63%). The sensitivity of the assay at 90% binding was 20 pg mL^{-1}.

Statistical analysis

Data were aligned to the day of mating on the individual profile. A composite profile was established after aligning data to the day of parturition and grouping it in monthly intervals. Data for the composite profile were presented as means ± SE, using pooled standard deviation, and then presented from month 1 to 15 of gestation because the gestation period was 15 months. The Shapiro-Wilk W test was used to determine whether data sets for each animal and for each phase of pregnancy were normally distributed. Analysis of variance was used to evaluate differences in mean 20α-progestagen concentrations during each phase of gestation and between animals. Student's *t*-test was used to investigate differences between mean hormone concentrations in non-pregnant animals and those sampled at different phases of gestation. The probability distribution of the sample mean (i.e. ± 1.96 × SE) was used to calculate the 95% confidence interval (CI) whereas the probability distribution of the original data (i.e. ± 1.96 × SD) was used to calculate the 95% upper tolerance limit of 20α-progestagen concentrations for non-pregnant females. Statistical analyses were performed using a statistical software package (Statistix for Windows, Analytical Software, Tallahassee, FL, USA).

Evaluation of the predictive values for pregnancy diagnosis, of individual test results, was performed by analysis of the 'specificity' and 'sensitivity' as described by Altman (1991). Specificity is defined as the proportion of negatives that are correctly identified by the test, and sensitivity is the proportion of positive results correctly identified by the test.

Results

Analysis of matched serum and faecal samples

The measurement of serum progesterone and faecal 20α-progestagens in paired serum and faecal samples (Fig. 1) showed that there was a strong relationship between levels of circulating progesterone and faecal progesterone metabolites ($r = 0.84$, $n = 15$, $P<0.001$), which suggests that faecal 20α-progestagens accurately reflects plasma progesterone concentrations in wild black rhinoceros cows. Serum P4 concentrations varied between 0.2 and 6.6 ng mL^{-1} and faecal 20α-progestagens concentrations ranged from 66 to 8410 ng g^{-1}.

Gestation length

In the wild, gestational length in two females was 456 days and 453 days. Another wild female had a gestation length of approximately 450 days, based on her hormonal profile.

Measurement of faecal 20α-progestagens during pregnancy

The individual profile obtained for one wild female (Fig. 2) showed cyclic fluctuations in faecal 20α-progestagen concentrations before pregnancy, and mating activity was associated with a 5-day period of low progestagen concentrations. Within 5 days of mating, concentrations of faecal 20α-progestagen increased markedly and remained elevated for 23 days before decreasing. Concentrations of 20α-progestagen then increased substantially after day 101 of the profile, and declined to pre-mating levels within 4 days following parturition.

Because progestagen concentrations increased markedly after the third month of pregnancy, the gestation period was

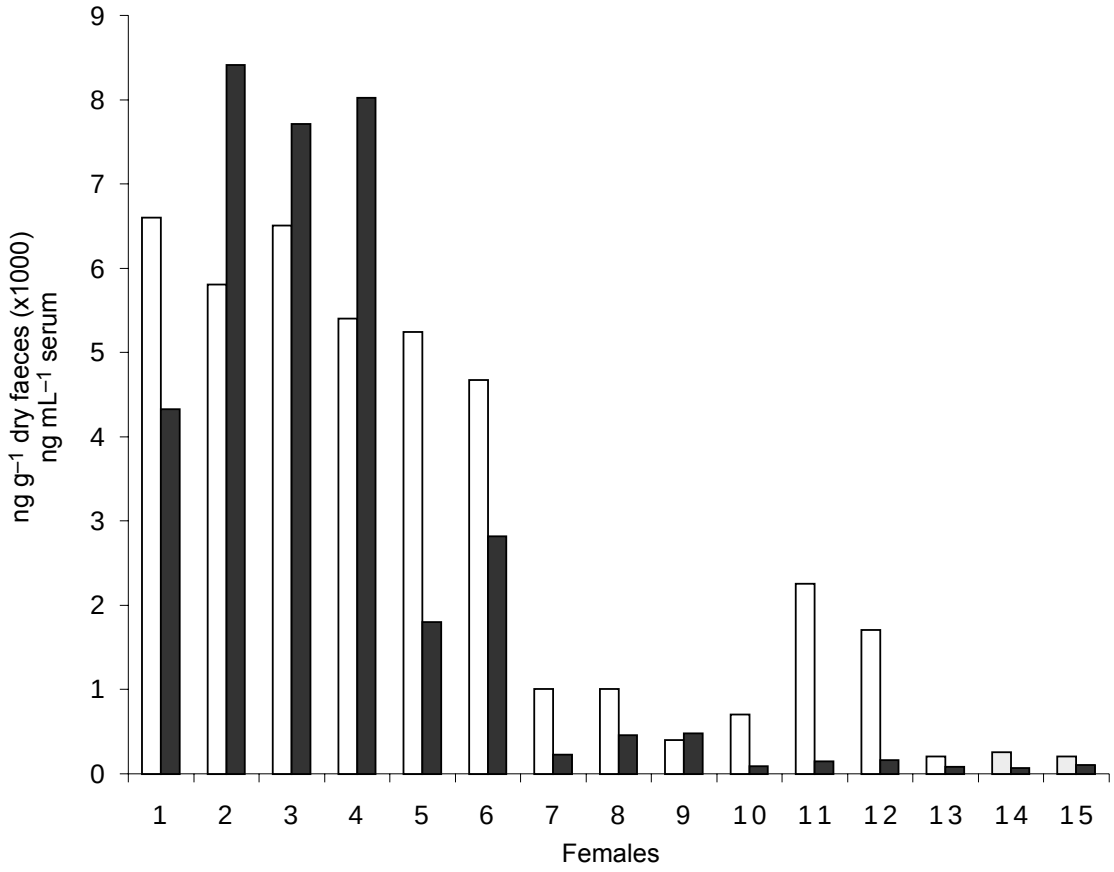

Fig. 1. Relationship between (■) faecal 20α-progestagen and (□) serum progesterone concentrations in paired faecal and serum samples collected from wild black rhinoceros females ($n = 15$). The first six females were known to be pregnant at the time of sampling.

divided into early, mid, and late phases, which included months 1–3, 4–12 and 13–15, respectively, of gestation. Mean 20α-progestagen concentrations during each phase of pregnancy were not significantly different ($P = 0.10$) between the six wild study animals and their data were therefore combined in Table 1. Mean concentrations in each different phase of pregnancy were significantly higher ($P<0.0001$) than in non-pregnant animals.

A composite profile of faecal 20α-progestagen concentrations during six pregnancies is illustrated in Fig. 3. Mean 20α-progestagen concentrations during the first month (1359 ± 186 ng g^{-1}) and third month (2000 ± 265 ng g^{-1}) of gestation were significantly higher ($P = 0.014$ and $P = 0.002$ respectively) than non-pregnant concentrations (800 ± 58 ng g^{-1}), but mean concentrations during the second month of pregnancy (1106 ± 23 ng g^{-1}) did not differ significantly ($P = 0.08$) from non-pregnant concentrations. Between months 4 and 15 of gestation, mean concentrations remained between 5 and 10 times greater than non-pregnant concentrations.

The 95% upper tolerance limit for 20α-progestagen concentrations in non-pregnant females was 2005 ng g^{-1}. Using 2000 ng g^{-1} as a threshold, all individual samples collected from the six wild females were tested retrospectively, to determine the percentage of false negatives and false positives that would occur when diagnosing pregnancy from progestagen analysis in a single sample (Table 2). On this basis, a positive diagnosis of pregnancy or non-pregnancy could be made with 96.5% and 76.8% confidence, respectively. Because these predictive percentages depend on the prevalence of pregnant samples in the test, the specificity and sensitivity of the test represent a better indicator of reliability. The probability of a false positive or false negative occurring would be 4.9 and 17.6%, respectively. However, after the third month of gestation, the sensitivity of the test increased to 97.6%, thus reducing the probability of a false negative to 2.4%.

Discussion

Our results demonstrate that measurement of concentrations of 20α-progestagens in faeces provided a reliable indicator of pregnancy in free-ranging black rhinoceros.

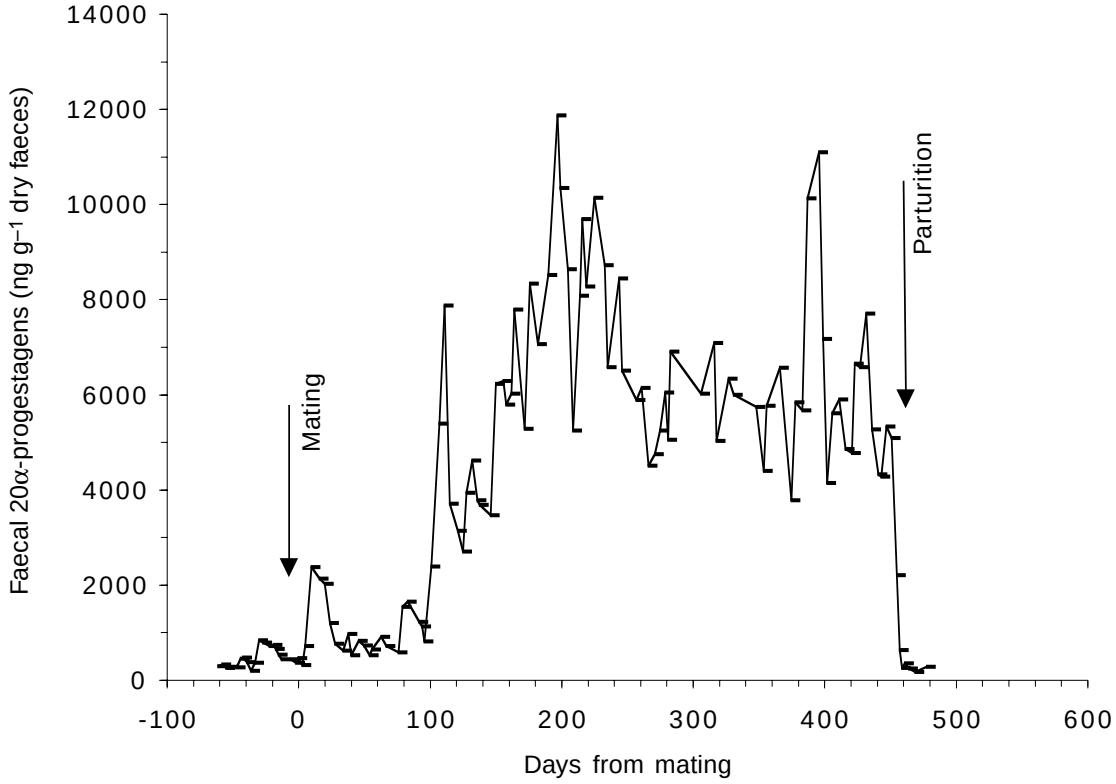

Fig. 2. Concentrations of immunoreactive 20α-progestagens in faecal samples of a wild black rhinoceros female during an oestrous cycle and subsequent pregnancy.

Only one of our study animals was radio-collared and, despite the immense size of the main study area and the presence of a large number of black rhinoceros, we were able to collect faecal samples from known individual females on a very regular basis. Black rhinoceros in the Save Valley Conservancy live in dense bush habitats and are very wary of human presence, which they can rapidly detect due to their very developed sense of smell and hearing (Shenkel and Shenkel-Hulliger 1969). When disturbed, these animals will either run away or become aggressive towards humans, which might result in a change of their normal activity pattern (Garnier, unpublished data). For these reasons, most of our direct observations only lasted a few minutes and it was impossible to remain in the vicinity of an animal for long enough to observe the occurrence of defecation, as has been done in other studies with free-ranging wildlife (Kirkpatrick *et al.* 1990, 1991, 1992, 1993; Messier *et al.* 1990; White *et al.* 1995). However, the solitary habits of black rhinoceros, combined with the unique ability of local game scouts to identify individual females from their footprint, allowed us to find known females and to sample their fresh dung pile without disturbing the animals.

The rapidity and success in finding a sample from a given individual varied greatly with the animal, the time of the year and the presence of a radio-collar. In general, females were much easier to find during the dry season, when they tend to remain nearby known water points and become inactive as soon as the temperature becomes too hot. The radio-collared animal could always be located when needed, in a time rarely exceeding 2 h. By contrast, it could take up to 6 h to find a sample from a non-collared animal. Either of the two dung piles that had been dropped by a female before finding her was collected (Garnier *et al*. 1998).

The correlation between serum progesterone and faecal 20α-progestagen indicate that in wild black rhinoceros females the measurement of faecal progestagen using a

Table 1. Faecal 20α-progestagen concentrations (ng g^{-1} dry faeces) in wild black rhinoceros (n = 6) at various stages of pregnancy and non-pregnancy

Reproductive status*	Mean	SE	n	95% confidence limits†
Non-pregnant	800	58	111	684–919
Early pregnancy	1417^{A}	131	57	1153–1680
Mid-pregnancy	6410^{A}	261	164	5895–6925
Late pregnancy	7243^{A}	472	47	6293–8192

*Non-pregnant, samples were collected one to two times per week during the 3 months preceding gestation, or between 4 to 7 months after parturition; early pregnancy, months 1–3 of gestation; mid-pregnancy, months 4–12 of gestation; late pregnancy, months 13–15 of gestation. †Mean ± 1.96 × SE. ^{A}P<0.05 when compared with non-pregnant concentrations.

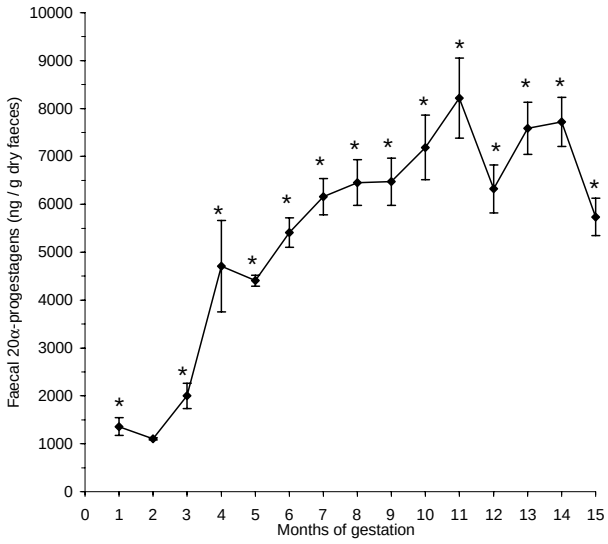

Fig. 3. Mean concentrations of faecal 20α-progestagens (± SE; n = 10–25 samples per point) during six pregnancies in wild black rhinoceros. *P<0.05 when compared with non-pregnant concentrations.

20α-OHP EIA provides a valid indirect indicator of ovarian activity. Although the nature of the immunoreactivity was not determined in the present study, previous studies using high-performance liquid chromatography have shown that 5α- and 5β-reduced pregnanes containing a 20-oxo or 20α-hydroxyl group are the main progesterone metabolites in faecal extracts collected from both captive and wild black rhinoceros (Patton *et al.* 1996; Schwarzenberger *et al.* 1996*b*), whereas 20α-OHP was a major progesterone metabolite in black rhinoceros urine (Hindle *et al.* 1992). Serum progesterone concentrations were similar in a captive female (Berkeley *et al.* 1997), but much lower than those reported by Kock *et al.* (1991) in wild females. Such differences are probably linked to differences in cross-reactivities of antibodies used.

Table 2. Characteristics of the pregnancy diagnosis test using faecal 20α-progestagen measurement in a single sample collected from wild black rhinoceros

Each unit tested represents one faecal sample. All samples tested were collected from the study animals (n = 6).

	Known status		
Predicted status	Not pregnant	Pregnant	Total
Not pregnant*	156	47	203
Pregnant†	8	221	229
Total	164	268	

Positive predictive value: 221/229 = 96.5%; negative predictive value: 156/203 = 76.8%; sensitivity: 221/268 = 82.4% (for definition see text); specificity: 156/164 = 95.1% (for definition see text).
*Faecal 20α-progestagen value <2000 ng g^{-1}; †faecal 20α-progestagen value ≥2000 ng g^{-1}.

The lengths of gestation periods obtained in this study compare favourably with previous observations in the wild (446 and 478 days, Goddard 1967; 455 days, Hall-Martin and Penzhorn 1977). These records involved the eastern subspecies *Diceros bicornis michaeli*, but no difference was found in captive black rhinoceros females between the eastern and southern subspecies (Schwarzenberger *et al.* 1993, 1996*b*).

The individual and composite profile of faecal 20α-progestagen concentrations in wild females resemble those reported in captive black rhinoceros (Schwarzenberger *et al.* 1993, 1996*b*; Berkeley *et al.* 1997). On the composite profile, mean pregnancy concentrations of 20α-OHP were significantly higher than non-pregnant concentrations as early as the third month of gestation. A similar scale of difference between pregnant and non-pregnant animals was found in previous studies (Schwarzenberger *et al.* 1993; Berkeley *et al.* 1997). Faecal progestagen levels in pregnant females were also comparable between wild and captive animals (Garnier, unpublished data), and therefore were not affected by variations in dietary fibre content. In captive baboons, increased dietary fibre was found to have a negative effect on progestagens excretion (Wasser *et al.* 1993).

The marked increase in concentrations after the third month of gestation probably reflects the onset of placental steroid production (Schwarzenberger *et al.* 1993). In horses, the placenta becomes the sole source of progestagens after the corpora lutea have regressed, secreting 5α-pregnanes (Holtan *et al.* 1975; Schwarzenberger *et al.* 1991). These progesterone metabolites have also been identified in black rhinoceros faeces (Patton *et al.* 1996; Schwarzenberger *et al.* 1996*b*).

The evaluation of faecal 20α-progestagen measurement as a diagnostic test in wild black rhinoceros showed that it was very accurate when repeated samples were collected from one female. When collecting only one faecal sample from a female, the sensitivity of the test was 97.6% after the third month of pregnancy. To minimize the chances of confusing pregnancy with a non-pregnancy stage, it is therefore suggested that weekly samples be collected for 4–6 weeks when testing a wild female. Should the results confirm that the female is pregnant, it would then be useful to keep collecting monthly samples in order to ensure that the pregnancy is carried to term. If pregnancy is not confirmed, the same protocol of weekly sample collection could be repeated at a later date, or a more frequent sample collection could be attempted (two or three samples per week) in order to identify oestrous cycles.

By being able to diagnose pregnancy remotely in individual black rhinoceros, surveillance can be increased around females in order to detect the occurrence of parturition and to monitor the newborn calf's health. This is particularly helpful because black rhinoceros females become increasingly difficult to find around the time of parturition and hide their

calves during the first few weeks post partum, thus rendering difficult the detection of perinatal mortality if the female's reproductive status is unknown (Garnier, unpublished data). The detection and monitoring of pregnancy can also enable the detection of fetal loss in individual females. This, in turn, will help to detect subfertile conditions and to determine the effects of management techniques, such as immobilization and translocation, on pregnancy. Such information is needed because adult female black rhinoceros have been found to be the easiest to translocate, when compared to black rhinoceros of different age classes or sex groups (P. Morkel, personal communication).

At a demographic level, the precise evaluation of pregnancy rates and birth rates will allow for the accurate assessment of fertility rates in wild black rhinoceros populations and for a rapid detection of changes in trends. By detecting lowered fertility rates, which can be linked to density-dependent effects, immediate and appropriate management decisions can then be made. These include the removal and translocation of surplus animals between breeding nuclei, decisions which need to be based on a sound knowledge of the individual's fertility status. Considering the scale of the costs and logistics associated with black rhinoceros management operations, this will greatly optimize management efficiency.

Conclusion

These results demonstrate that non-invasive diagnosis of pregnancy is possible in wild black rhinoceros females by using faecal progestagen analysis. Pregnancy could be diagnosed from the measurement of progestagens in a single sample, with less than a 5% risk of a false prediction after the third month of gestation.

From a conservation point of view, the non-invasive diagnosis of gestation in the wild will undoubtedly offer a new perspective for managing black rhinoceros populations *in situ*. The efficiency of management techniques in wild populations can be increased through the accurate knowledge of an animal's reproductive status and a better understanding of the extrinsic parameters affecting reproductive health can be gained.

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