Rapid colour-change pregnancy test for rhinoceros using faeces

Edith A. MacDonald[1,2,*], **Wayne L. Linklater**[1,3,4], **Karen J. Steinman**[1,5], **Nancy M. Czekala**[1,6]

[1]Conservation and Research for Endangered Species, Zoological Society of San Diego, 15600 San Pasqual Valley Road, Escondido, California 92027, USA

[2]*Present address:* Wellington Zoo Trust, 200 Daniel Street, Newtown, Wellington 6021, New Zealand

[3]*Present address:* Centre for Biodiversity and Restoration Ecology, School of Biological Sciences, Victoria University of Wellington, PO Box 600, Wellington 6021, New Zealand

[4]*Present address:* Centre for African Conservation Ecology, Department of Zoology, Box 77000, Nelson Mandela Metropolitan University, Port Elizabeth, Eastern Cape 6031, South Africa

[5]*Present address:* Sea World & Busch Gardens Reproductive Research Center, Busch Entertainment Corporation, 500 SeaWorld Drive, San Diego, California, USA

[6]*Present address:* Papoose Conservation Wildlife Foundation, PO Box 575, Del Mar, California 92014, USA

ABSTRACT: Pregnancy is routinely determined by monitoring faecal progestogen levels in black and white rhinoceros, *Diceros bicornis* and *Ceratotherium simum*, respectively. However, current laboratory procedures are too slow and not practical in the field, and transporting samples to the laboratory may not be possible, may cause them to deteriorate, or result in delays such that events subsequent to sampling supersede the results. We modified current enzyme-immuno assays (EIA) methods to create a field test that is robust under field conditions, requires minimal technical equipment and expertise, and yields results on-site within 24 h. The field assay is a tri-colour test and pregnancy is determined visually. Faecal samples from 55 free-ranging white and 7 black rhinoceros were collected from the rectums of animals at capture and from fresh dung piles while the rhinoceros were temporarily housed in boma. The accuracy of the colour-change field test was confirmed by radio-immuno assay (RIA) of matched faeces and independent blood serum analyses for 23 pregnant and 39 non-pregnant rhinoceros. This field pregnancy test is likely to be appropriate for other animals, such as elephants, that have high and sustained levels of progestogen during pregnancy.

KEY WORDS: Enzyme-immuno assay · *Diceros bicornis* · *Ceratothirum simum* · Reproductive monitoring · Progestogen

INTRODUCTION

Laboratory techniques for detecting pregnancies from elevated faecal progestogen using enzyme- (EIA) and radio- (RIA) immuno assay procedures are well established for black and white rhinoceros, *Diceros bicornis minor* and *Ceratotherium simum*, respectively (Kock et al. 1991, Schwarzenberger et al. 1993, Czekala & Callison 1996, Schwarzenberger et al. 1996, Berkeley et al. 1997, Garnier et al. 1998, Patton et al. 1999, Graham et al. 2001). However, current laboratory methods are not easily transferred and conducted in a field setting, although rapid on-site pregnancy tests are required to assist in field research and expedite management (e.g. during capture and translocation, Linklater et al. 2006).

RIAs require equipment and supplies that are not always feasible in the field, such as radioactive reagents and scintillation counters. Moreover, managers and field rangers working with endangered species only rarely have experience and expertise in immuno-assay techniques. Preserving samples in the field by freezing and transporting them to the labora-

tory is also sometimes impractical, particularly from remote locations where many endangered species live. Although current EIAs are more feasible than RIAs at field sites, they still necessitate a clean environment and expensive plate readers requiring a reliable electricity supply. Thus, the next advance in pregnancy testing techniques for endangered species must be colour-change tests, like those readily available for humans and domestic stock (Henderson & Eayrs 2004), that can be conducted rapidly on-site with minimal expertise to contribute timely reproductive information during management and research.

Compared to other endangered species, the reproductive physiology of African rhinoceros is well known (Roth 2006). Black rhinoceros have an estrus cycle of 24 to 26 d, with progestogen concentrations during the luteal phase 1.3 to 2× higher than during the interluteal phase. Progestogen concentration rises after conception and is significantly (4 to 10×) higher after the third month of gestation than during the luteal phase (Schwarzenberger et al. 1996, Berkeley et al. 1997). Gestation is approximately 460 d (approx. 15 $\frac{1}{3}$ mo) for black rhinoceros (literature summarised in Linklater 2007).

Two cycle lengths have been identified in captive white rhinoceros (Patton et al. 1999, Graham et al. 2001). One is approximately 35 d and the other of an approximate 2 mo duration. However, white rhinoceros suffer from a low reproductive rate in captivity (Hermes et al. 2006) and the longer 2 mo cycles are likely to be a captive aberration because wild white rhinoceros appear to only demonstrate approximately 35 d cycles (Ron R. Swaisgood pers. comm.). On average, progestogen concentration during the luteal phase is almost 4× greater than during the inter-luteal phase. Gestation averages 495 d (approx. 16 $\frac{1}{2}$ mo) for white rhinoceros (Linklater 2007) and progestogen concentration begins to rise between 1 and 3 mo post-conception and is significantly greater than luteal levels at approximately 3 to 4 mo gestation. Progestogen concentration continues to rise until the 7th month, when it is >100× higher than luteal values (Patton et al. 1999, Brown et al. 2001, Graham et al. 2001).

The purpose of the present study was to modify existing laboratory-based qualitative progestogen assays into a field-based qualitative EIA assay that is simple to perform, cost effective, rapid, and robust and reliable under field conditions. To this end, we describe a simple colour-change field pregnancy test using faeces and based on progestogen concentration and the known reproductive parameters of white and black rhinoceros. We trialled and replicated the test in the field to assess its accuracy under varying conditions and validated the results in the laboratory with traditional quantitative techniques.

MATERIALS AND METHODS

Animals and sample collection. The study animals included 55 white rhinoceros and 7 black rhinoceros endemic and free-ranging in Hluhluwe-iMfolozi Park (KwaZulu-Natal province, South Africa: 28° 51′ 29″ S, 31° 13′ 40″ E) that were part of a much larger management and research programme on rhinoceros translocation (i.e. Linklater et al. 2006). Subject animals were captured and temporarily housed in boma (robust enclosures for temporarily holding rhino prior to release) before transportation for release at another reserve during the period August–October, 2004. Length of stay in the boma varied from 5 to 10 wk, which also corresponds approximately to at least one oestrous cycle (25 and 35 d for black and white rhino, respectively).

Approximately 150 g samples of faeces were gathered directly from the rectum while all rhino were immobilized for capture and a blood sample taken from 18 of the white rhino, and all 7 of the black rhino by venupuncture. Subsequent faecal samples were collected weekly from fresh piles of dung while the rhino were in boma as close to Days 7, 14, 21, 28 and 35 from capture as possible. The collection regime differed for white and black rhinoceros due to differences in how the 2 species were housed. White rhino were housed in groups in large boma, making the collection of fresh samples from known animals more difficult. Only the dung piles from observed defecations were sampled for white rhino. Black rhinos were housed solitarily and, thus, attributing samples to individuals and collecting them was easier. Dung was taken from the middle of the largest boli, and duplicate samples (one for field analysis and one for laboratory analysis) were placed in plastic specimen cups and frozen immediately in a vehicle-based freezer.

EIA. Samples remained frozen until analysis. After thawing, 0.5 g of wet feces was weighed in plastic tubes (Sarstedt). A total of 5 ml of an 80% methanol/water solution was added, capped and shaken vigorously by hand for 30 s and incubated overnight at room temperature (25°C). The next morning, samples were shaken by hand again and allowed to settle for 10 min. A faecal dilution was made by removing 10 µl from the top of the vial to avoid any foliage and adding it to 1 ml of distilled H_2O (dH_2O) in a glass test tube.

The diluted faecal samples were analyzed using a monoclonal progesterone antibody EIA technique. Antibody and enzyme conjugate solutions were stored frozen in a freezer until used for the assays. Once thawed, they were stored in a refrigerator along with the other assay buffers. The plates were kept in the same thick Styrofoam box used in the laboratory during the development of the field assay. For the EIA, 96-well flat bottom microlitre plates (Immulon 1, Dynex

Technologies) were used. Eighty μl of a 1:100 progesterone antibody (antibody CL425, C. Munro, University of California, Davis, CA) was diluted in 8 ml of a coating buffer (0.05 M, pH 9.6). Fifty μl of the solution was pipetted into each well and incubated overnight at 4°C (for cross reactivity of the CL425 antibody, see Graham et al. 2001). After incubation, plates were washed twice with a solution (0.04 % Tween 20) and shaken dry. The standard EIA 8 point quantitative curve (250 to 1.95 pg) which had been previously used in the laboratory was reduced to 3 points, each with a distinct colour. Each colour (clear, light blue, or dark blue) corresponded to a hormonal threshold level indicative of a different reproductive stage in a rhinoceros (pregnant, luteal, or follicular, respectively). Thus, the EIA was adapted so samples were classified to reproductive status without quantification of hormonal concentration. This modification was possible because hormonal levels in rhinoceros increase at least 2-fold from the follicular to luteal stage and by as much as 2 orders of magnitude during pregnancy. Blind testing in the laboratory of the same samples using the 3-standard classification method correctly identified samples as pregnant, luteal, or follicular at the same rate compared to quantitative analysis using the 8 point standards. Thus, non-synthetic standards representing pregnant, luteal and follicular phase were used (see Table 1 for comparison of EIA and RIA values to the field colour change). Fifty μl of the faecal dilutions and standards were added in duplicate followed by the addition of 50 μl enzyme conjugate solution (1:20 000 in a phosphate buffer 0.1 M, pH 7.0; progesterone horseradish peroxidase, P-HRP, C. Munro, University of California, Davis, CA). After 2 h room temperature incubation, plates were emptied, rinsed with the wash solution, and shaken dry. One hundred μl of a 3,3′,5,5′-tetramethylbenzidine (TMB)/ phosphate citrate solution was added (1 TMB tablet; Sigma-Aldrich) then dissolved into 10.5 ml of a phosphate-citrate buffer created by one capsule (Sigma-Aldrich) diluted in 100 ml of dH_2O. Plates were incubated at room temperature in the dark for 30 min and then visually read independently by 2 researchers.

Corroboration using faecal and serum RIA. The accuracy of the colour-change field test was confirmed for faeces using a RIA that employed the same progesterone antibody (Patton et al. 1999) at the Center for Research on Endangered Species at the San Diego Zoo. Validation parameters for those assays are reported in Patton et al. (1999). Independent serum analysis was also conducted by the University of Pretoria, Veterinary Wildlife Service.

Table 1. Concentrations of faecal progestogen from quantitative enzyme-immuno assays (EIA) and radio-immuno assay (RIA) that correspond to the colours derived from the qualitative field-based EIA described

Reproductive status	Field EIA colour	EIA progestogen range (ng ml⁻¹)	RIA progestogen range (ng g⁻¹)
Follicular	Dark blue	<18	<200
Luteal	Light blue	18–250	200–1000
Pregnant	Clear	>250	>1000

RESULTS

A clear colour reading indicated high concentrations of faecal progestogen, i.e. positive for pregnancy. Luteal and early pregnancy samples (i.e. less than approximately 4 mo post-conception) were light blue, and follicular samples were dark blue (Table 1). Fig. 1 graphically depicts a schematic of faecal progestogen profiles during oestrous cycling and pregnancy and the corresponding colours that result from our pregnancy test.

Differentiation between luteal and early pregnancy was possible when the 4 consecutive weekly samples were reviewed. For rhino in early pregnancy the reading remained light blue or showed a trend from light blue to clear as progestogen concentration increased. In contrast, a non-pregnant but cycling female displayed light blue samples during the luteal phase and, within approximately 2 wk, displayed dark blue readings indicating the follicular phase (Fig. 2). Thus, a female with 4 consecutive weekly light blue samples or
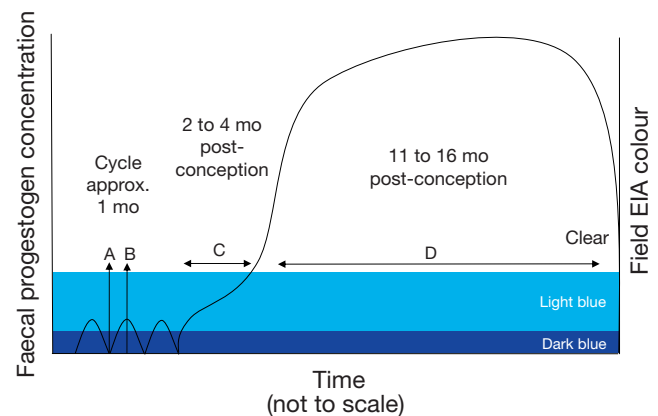

Fig. 1. Faecal progestogen concentrations exhibit a temporal pattern, with low values during the follicular phase (A), increased values during the luteal phase (B) that are sustained through early pregnancy (C), and significantly higher values during later gestation (D). The field pregnancy test is dark blue for samples in the follicular phase, light blue during the luteal phase and early pregnancy, and clear during later pregnancy. See Schwarzenberger et al. (1996) and Patton et al. (1999) for detailed analysis of cycle and pregnancy length and corresponding progestogen concentrations
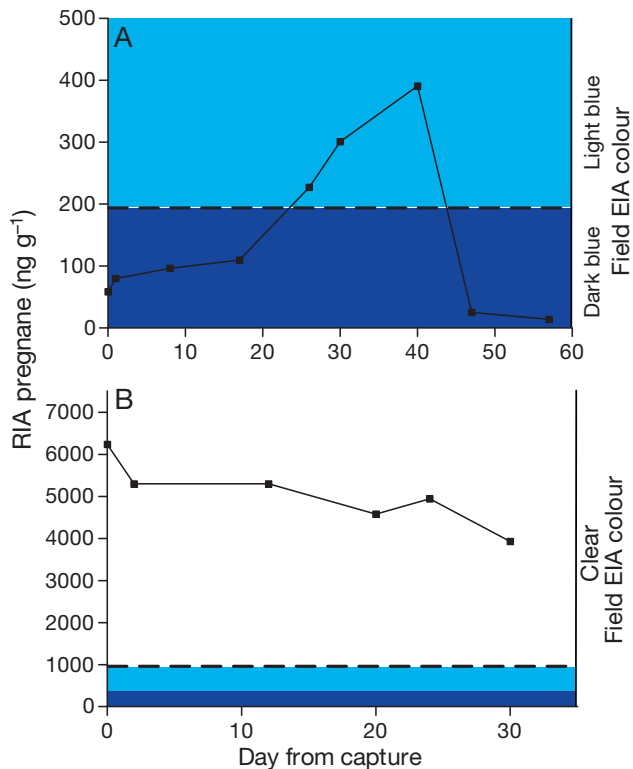
Fig. 2. *Diceros bicornis*. (A) Results shown for black rhino B3 from capture (Day 0) and the subsequent week samples while captive in boma. Left *y*-axis displays the quantitative results of the radio-immuno assay (RIA) analysis conducted in the laboratory. Right *y*-axis indicates the threshold (horizontal dashed line) for visible identification of samples as luteal (light blue) or interluteal (dark blue) by the field enzyme-immuno assays (EIA) colour test. Based on her initial dark blue readings, followed by light blue readings, and a return to dark blue readings, B3 was categorized as non-pregnant and cycling. Laboratory RIA analysis was in accordance with this diagnosis. (B) Results shown for black rhino C2. Right *y*-axis indicates the threshold (horizontal dashed line) for visible identification of samples as pregnant (clear), luteal (light blue) or interluteal (dark blue). Based on her consecutive clear readings in the field, she was categorized as pregnant. Laboratory RIA analysis was in accordance with this diagnosis

a tendency to clear readings could be identified as being in early pregnancy, while a female with alternating light and dark blue samples over the study time frame was identified as cycling.

Classification of black and white rhinoceros reproductive state using the overnight incubation colour field EIA test corresponded 100% to laboratory RIA analysis, with no false positives or negatives. Twenty-one white and 2 black rhinoceros were pregnant and 34 white and 5 black rhinoceros were not pregnant. The independent serum results also matched the field tests: 11 of the blood-sampled white rhino were categorized as pregnant and 5 black rhino as not pregnant.

Pregnancy in 4 females (2 white and 2 black rhino) resulted in miscarriage, with visible foetuses being expelled overnight and found by staff in the morning. Faecal progestogen concentration remained at pregnancy levels for up to 2 d after the miscarriages but subsequent weekly samples were at interluteal levels. Similarly, one white rhinoceros gave birth while in the boma. She still had elevated faecal progestogen concentrations 2 d after the birth but interluteal levels for the next available sample (6 d after birth).

DISCUSSION

More rapid, inexpensive and less technical diagnosis of pregnancy is required at remote field sites for research and management purposes. We successfully created a simple and robust field pregnancy test that uses faeces and was based on a modification of current laboratory tests. The new technique does not require the large expense of a plate reader, is not dependent on electricity, and reduces the need for dangerous reagents (e.g. sulphuric acid) that cannot always be transported or obtained at the field site. Moreover, the technique can be conducted on-site, thus reducing the delays and logistical difficulties of transporting samples to distant laboratories, particularly when samples need to remain cool. Moreover, in the years subsequent to this trial we have trained field-based assistants, without previous reproductive laboratory experience, to successfully conduct these tests. The field test's accuracy was confirmed independently, and its responsiveness to changes in the reproductive condition of individual animals through time (e.g. parturition and pregnancy termination) demonstrated its utility. In the application of this technique we caution, however, that while this test is appropriate for detecting pregnancy, it is not appropriate for predicting parturition or termination because of the time required for boli to transit the gut and be deposited as faeces and the overnight incubation required of the assay. Thus, a faecal test of this nature is inadequate as a predictor of imminent birth or termination (i.e. within 3 to 5 d).

Using faeces, rather than serum or saliva, to test for pregnancy is better suited to the management of endangered species like rhinoceros and logistically simpler under field conditions than other methods. The risks and cost of collecting faecal samples are considerably less than for blood or saliva samples (Czekala & Callison 1996, Berkeley et al. 1997, Brown 2000, Graham et al. 2001) but the opportunities are greater, particularly for free-ranging and wild animals. Moreover, tests that use faeces allow managers to routinely collect samples and monitor for changes in reproductive state amongst a few select individuals (i.e. termination

of pregnancy, acyclic, and cycle resumption post-partum) or even to determine and monitor fecundity rates across large populations, where knowing reproductive rates between populations can assist in appropriately targeting conservation effort and resources for greatest gain. This new test makes it possible for detailed reproductive monitoring to occur on-site both *in situ* as well as *ex situ* within a time frame that is appropriate for informing management decisions.

Lastly, this field pregnancy test may be suitable for the many other species that have significant and sustained levels of progestogen during pregnancy, such as the other 3 critically endangered rhinoceros species in Asia, elephants (Freeman et al. 2007) and okapi (Schwarzenberger et al. 1996).

LITERATURE CITED

Berkeley E, Kirkpatrick J, Schaffer N, Bryant W, Threlfall W (1997) Serum and fecal steroid analysis of ovulation, pregnancy, and parturition in the black rhinoceros (*Diceros bicornis*). Zoo Biol 16:121–132

Brown JL (2000) Reproductive endocrine monitoring in elephants: an essential tool for assisting captive management. Zoo Biol 19:347–367

Brown J, Bellem A, Fouraker M, Wildt D, Roth T (2001) Comparative analysis of gonadal and adrenal activity in the black and white rhinoceros in North America by noninvasive endocrine monitoring. Zoo Biol 20:463–486

Czekala NM, Callison L (1996) Pregnancy diagnosis in the black rhinoceros (*Diceros bicornis*) by salivary hormone analysis. Zoo Biol 15:37–44

Freeman E, Abbondanza F, Brown J (2007) Development of a field kit for monitoring hormone activity in free-living African elephants (*Loxodonta africana*). XXI Annu Conf Soc Conserv Biol. International Society for Conservation Biology, Port Elizabeth

Garnier JN, Green DI, Pickard AR, Shaw HJ, Holt WV (1998) Non-invasive diagnosis of pregnancy in wild black rhinoceros (*Diceros bicornis minor*) by faecal steroid analysis. Reprod Fertil Dev 10:451–458

Graham L, Schwarzenberger F, Mostl E, Galama W, Savage A (2001) A versatile enzyme immunoassay for the determination of progestogens in feces and serum. Zoo Biol 20: 227–236

Henderson KM, Eayrs K (2004) Pregnancy status determination in mares using a rapid lateral flow test for measuring serum oestrone sulphate. NZ Vet J 52:193–196

Hermes R, Hildebrandt T, Walzer C, Goritz F and others (2006) The effect of long non-reproductive periods on the genital health in captive female white rhinoceroses (*Ceratotherium simum simum, C.s. cottoni*). Theriogenology 65: 1492–1515

Kock N, Morton D, Kock M (1991) Reproductive parameters in free-ranging female black rhinoceroses (*Diceros bicornis*) in Zimbabwe. Onderstepoort J Vet Res 58:55–57

Linklater WL (2007) Translocation reverses birth sex ratio bias depending on its timing during gestation: evidence for the action of two sex allocation mechanisms. Reprod Fertil Dev 19:831–839

Linklater W, Flammand J, Rochet Q, Zekela N and others (2006) Preliminary analyses of the free-release and scent-broadcasting strategies for black rhinoceros re-introduction. Ecol J 7:26–34

Patton ML, Swaisgood RR, Czekala NM, White AM and others (1999) Reproductive cycle length and pregnancy in the southern white rhinoceros (*Ceratotherium simum simum*) as determined by fecal pregnane analysis and observations of mating behavior. Zoo Biol 18:111–127

Roth T (2006) A review of the reproductive physiology of rhinoceros species in captivity. Int Zoo Yearb 40:130–143

Schwarzenberger F, Francke R, Goltenboth R (1993) Concentrations of faecal immunoreactive progesten metabolites during the oestrus cycle and pregnancy in the black rhinoceros (*Diceros bicornis michaeli*). J Reprod Fertil 98: 285–291

Schwarzenberger F, Tomasova K, Holeckova D, Matern B, Mostl E (1996) Measurement of fecal steroids in the black rhinoceros (*Diceros bicornis*) using group-specific enzyme immunoassays for 20-oxo-pregnanes. Zoo Biol 15: 159–171