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Assessment of reproductive status of the black rhinoceros (*Diceros bicornis*) in the wild

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Synopsis

There is a need for the development of techniques in monitoring the reproductive performance of the black rhinoceros in the wild, particularly as a tool for improving breeding performance, and in cohesive management of the small protected populations that remain in Kenya. The priorities here for long-term propagation of this endangered species are easy detection of oestrus and pregnancy in females and assessment of breeding status in males. The social organization and reproductive physiology of the indigenous, protected black rhinoceros population of Ol Ari Nyiro ranch, Laikipia, are being monitored to this end. The home range movements and behaviour of resident rhinoceros have been recorded and urine samples have been collected from known individuals and assayed for hormone metabolites in order to provide a non-invasive indicator of breeding status.

Methods of collection of urine from free-living rhinoceros are described. Pregnanediol-3-glucuronide immunoreactivity was measured in urine from females by a simplified enzyme assay procedure, showing the potential of this as a field test for the detection of mid–late pregnancy. Urinary oestrone-3-sulphate did not reflect reproductive status in males, although a positive association of body size and home range size was found. Levels of urinary immunoreactive testosterone in males were extremely low and did not correlate with breeding status. Subsequent chromato-

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graphic analysis showed testosterone to be completely undetectable but revealed an 'androstane-tiol-one' as the major androgen metabolite. The potential for the use of endocrine monitoring techniques in the black rhinoceros is discussed, particularly in relation to the potential need to be able to transfer genetic material between small rhinoceros populations as part of their long-term management.

Introduction

The number of black rhinoceros remaining in Kenya is estimated to be in the region of 400. Much money and effort is being spent on measures to protect and manage the few viable black rhinoceros populations remaining in Kenya within special sanctuaries or reserves where their interests are paramount. The need for sanctuaries was spelt out clearly over two decades ago (Ritchie 1963); the Kenya black rhinoceros population has dropped by *c.* 98% since then.

The remnant black rhinoceros populations in Kenya are small and fragmented, and successful management and breeding in the long term will require the ability easily to assess their reproductive status. Firstly, there is the need for a reliable determination of pregnancy in female rhinoceros. Apart from the difficulty of observing black rhinoceros in their favoured habitat of dense bushland, pregnant females do not become noticeably gravid even during the later stages of pregnancy. Secondly, an indicator of breeding status in mature male rhinoceros would be tremendously useful for long-term management of black rhinoceros under conditions where the movement of breeding animals between populations will be essential for their genetic and demographic health. Breeders need to be easily identified, and the total number of adult rhinoceros breeding in a given population assessed in order to estimate its genetically effective population size (Lande & Barrowclough 1987).

To date, the only available method for identifying and assessing reproductive status has been observation. Even here, reliable information on breeding behaviour, particularly mating, is extremely sparse, largely because of the difficulty of obtaining frequent observations of black rhinoceros in dense bush habitat.

Goddard (1966) and Schenkel & Schenkel-Hulliger (1969) recorded most of the little information available on the breeding behaviour of black rhinoceros. Apart from mating, the breeding and dominance behaviours in males include consorting with oestrous females around the time of mating, mate-guarding, a high frequency of spray urination, scraping hind feet through communal middens in the manner of white rhinoceros (Owen-Smith 1975), redirected activity in the form of destruction of bush (which is particularly associated with urination and defecation), and tracking the scent trails of oestrous females (Schenkel & Schenkel-Hulliger 1969; G.W. Frame pers. comm.).

Female rhinoceros in oestrus urinate frequently, so leaving scent trails (Goddard 1966), dribbling urine down their hind quarters which dries to form a white streaky deposit around and beneath the vulva. Together with a swollen vulva, and the close attentions of males, these are signs of oestrus. It appears that it is the manner of urination rather than a colour change in the urine that results in this white deposit. Previous observers have noted clear seasonal peaks in breeding behaviour, although black rhinoceros appear to be able to mate at any time of year (Ritchie 1963; Schenkel & Schenkel-Hulliger 1969; Mukinya 1973; Hall-Martin 1986).

Since the bush habitat in Laikipia is particularly dense, so that typically only four to six sightings of rhinoceros are made per week, and there is a pressing need to establish reproductive data on this population (only three matings have been observed on the ranch in two years), an alternative method of obtaining data was needed.

A relatively simple method of establishing the identity of breeding animals, particularly males, within any rhinoceros population, based on the measurement of reproductive hormones in urine, would be a potentially useful alternative or addition to chance observations of matings and/or of breeding behaviour, most of which must occur at night (Goddard 1967). Non-invasive assessment of reproductive status through urinary hormone analysis could therefore prove extremely useful as a management tool for determining which animals in a population were breeding, and in future development of methods of transferring genetic material through artificial breeding techniques as an alternative to the difficult, costly and often impractical exchange of breeding animals.

The practical advantages of using urine in reproductive studies on a variety of exotic species have been described by Hodges (1986) and Lasley (1985), although so far most of the available data are from captive animals, largely kept in zoos. The only reports on studies in the wild are from Poole, Kasman, Ramsay & Lasley (1984) on African elephants and from Andelman, Else, Hearn & Hodges (1985) on vervet monkeys. Similar studies in the wild have not previously been attempted on rhinoceroses owing both to the lack of opportunity for sample collection and to the absence of suitable laboratory techniques.

This paper describes initial efforts, firstly, to determine the feasibility of collecting urine from black rhinoceros under field conditions; secondly, to establish the usefulness of urinary hormone analysis for reproductive assessment; and thirdly, to introduce and simplify the methodology for use in field conditions, in particular to sample from the rhinoceros kept in sanctuaries in Kenya, which, given the areas of land enclosed for such purposes, are likely to have a mean holding potential of approximately 50 animals.

Methods

Study population

Data and samples were gathered from known individuals among an indigenous population of 47 black rhinoceros resident on Ol Ari Nyiro ranch, Laikipia, Kenya. This is an unfenced cattle and sheep range of *c.* 400 km^2 of lower highland bush country at an altitude of 1600–2000 m. The rhinoceros population, which occupies approximately one third of the ranch area, is protected by a private anti-poaching force, and is at medium density (Goddard 1967, 1970: *c.* 1 rhinoceros per 4 km^2).

Identification of rhinoceroses

Because of the difficult visibility in thick bush, rhinoceroses were mostly identified using the measurements and individual features of their footprints, in particular the width of the hind feet between the two side toes, in combination with the patterns of wrinkles in the base of the footpad, which are recorded in footprints in suitably fine, dry soil, and are unique to each animal (Fig. 1). Daily patrols of the extensive road network, water sources and salt licks on the ranch located sets of footprints which were tracked until the animal was sighted or located. Depending on the visibility, the identities of individual rhinoceros were confirmed from sex, horn size and shape, and notches and marks on the margins of the pinnae of the ears (Goddard 1966; Mukinya 1976).

Home range sizes

All locations of footprints of known animals were plotted on a 1:50 000 map of the ranch to an accuracy of ± 200 m. The data were analysed using the methods of Dixon & Chapman (1980) to yield home range probability contours. The areas within the 90% probability contours were used to measure range sizes: these gave best fit to the home range of each animal, excluding the effects of the occasional distant excursions that black rhinoceroses make from their home ranges.

Collection of urine

Urine was collected during tracking of a rhinoceros identified from its tracks or from subsequent sightings. Black rhinoceros in bush habitats typically urinate against shrubs that they pass, and may then scrape through the shrubs with their hind feet. Whenever fresh urine was detected during tracking (normally by its strong odour) and found on leaves it was collected. Fresh urine (about 1 h old or less) could be recognized because it was cloudy; a dark-coloured urine with a white precipitate would have been

Fig. 1. Photograph of the right hind footprint of a black rhinoceros, showing the wrinkle impressions from the base of the footpad used to identify individuals.

sitting for several hours. The urine was easily drawn up from the leaf axils using a 1 ml syringe (Fig. 2). The average volume collected was *c.* 0.5 ml, although much more could be obtained if a rhinoceros sprayed against a broad-leaved shrub.

The extent to which urine could be collected was influenced by the sex of individual rhinoceroses, creating a bias in sampling. Spray-urination (as described by Schenkel & Schenkel-Hulliger (1969)) is performed more by dominant males, which project atomized urine for several metres to their rear, and if this is directed onto a bush, and the droplets are deposited all over it, the urine is easy to collect.

Non-ritualized urination, which is the more usual method of females and subdominant males, results in a stream of urine directed down or to the

Fig. 2. Photograph of fresh urine being collected for hormone assay from the leaves of a shrub onto which a passing rhinoceros has spray-urinated.

rear, and not necessarily over a bush, and the urine is rapidly absorbed into the ground. This made it particularly difficult to obtain urine from females, which are more reluctant to spray-urinate against bushes than males, and will dribble urine at frequent intervals during oestrus. Of a total of 100 urine samples collected over 18 months in 1986–87, only 17 came from females. All urine samples collected in the field in Kenya were frozen and sent to the Institute of Zoology at London Zoo, where they were kept at –20°C without additives until analysis.

Urine samples were also collected from captive animals for comparative purposes. Samples were collected from a single female during the last six months of pregnancy, and from two females over the oestrous cycle. Samples were also collected from three adult males, all proven breeders. Collections were made during the early morning where possible, urine being drawn up from the floor with a syringe after deposition. Samples were frozen and centrifuged prior to assay.

Hormone analysis

Testosterone

Testosterone was measured by radioimmunoassay using a modification of the method previously described by Hodges, Eastman & Jenkins (1983). The antiserum (Steranti Research Ltd) was raised in a rabbit against

testosterone-3-CMO-BSA and showed maximum cross-reactivity of 52% against 5α dihydro-testosterone. Conjugated and unconjugated testosterone were measured with and without a hydrolysis step respectively prior to extraction and assay. Hydrolysis was achieved by overnight incubation with glucuronidase arylsulphatase (1000 units) at pH 5 and 37°C. Extraction (0.5–1.0 ml sample) was achieved with methanol: water primed C_{18} Seppak catridges using 100% methanol as eluting solvent. The extract was dried under nitrogen and reconstituted in buffer, ready for assay (mean ± s.e. recovery = 79 ± 3.4%) Sensitivity of the assay was 5 pg/tube or 80 pg/ml and inter- and intra-assay CVs were below 15%. The relationship between serial dilutions of rhinoceros urine and testosterone standards was variable: dilutions of some samples were parallel to the standards whereas dilutions of others were not. In all cases levels of testosterone were very low, producing a maximum displacement of binding of only approximately 30% (1.0 ml sample).

Conjugated oestrone

Oestrone conjugates were measured using a non-extraction assay according to the method described by Hodges & Eastman (1984) and Eastman, Makawiti, Collins & Hodges (1984). In brief, 0.1 ml of diluted urine (1:5 v/v) was incubated overnight at 4°C with 0.1 ml antiserum raised against oestrone-3-glucuronide-BSA (1:15 000 initial dilution) and 0.1 ml ^{3}H oestrone-3-sulphate as tracer (Sp.Act 48 Ci/mmol). Doubling dilutions of oestrone-3-sulphate standards were run over the range 2000–31.3 pg/tube. Cross-reactivities of the antiserum include 126% oestrone, 84% oestrone-3-glucuronide, 5.4% oestrone-17-sulphate, and 0.1% oestradiol-3-sulphate. Serial dilutions of male rhinoceros urine gave displacement curves parallel to those obtained with oestrone-3-sulphate standards. Assay sensitivity was 18.5 pg/tube or 0.92 ng/ml urine and inter- and intra-assay coefficients of variation were $< 10\%$.

Pregnanediol glucuronide

Pregnanediol glucuronide immunoreactivity was measured in unextracted samples using a microtitre plate enzyme assay described in detail and validated for use in the rhinoceros by Hodges & Green (in prep.). The assay utilizes an antiserum raised in a rabbit against pregnanediol-3-glucuronide-BSA and alkaline phosphatase conjugated to pregnanediol-3-glucoronide (Sauer, Foulkes, Worsfold & Morris 1986; Hodges, Green, Cottingham, Sauer, Edwards & Lightman 1988) as enzyme conjugate. Serial dilutions of rhinoceros urine gave displacement curves parallel to that obtained with standards. Cross-reactivity of the antiserum has been previously reported (Hodges & Green in prep.). Because of the absence of definitive identification of pregnanediol-3-glucuronide in black rhinoceros urine the true identity of

the hormone being measured cannot be verified. Accordingly, the results are expressed as pregnanediol glucuronide immunoreactivity. Assay sensitivity was 10 pg/well or 0.6 ng/ml and inter- and intra-assay CVs were below 15%.

Creatinine

The creatinine content of each urine sample was estimated to help compensate for differences in urine concentration and volume (Brand 1981; Hodges & Eastman 1984). Sensitivity of the assay was 0.1 mg creatinine and interassay coefficient of variation $< 10\%$. Hormone levels reported in this paper have been divided by urinary creatinine concentration and are thus expressed as mass (ng)/mg creatinine.

Results

Home range and body size

The home range sizes of six adult male black rhinoceroses are shown in Table 1, set against the width of their hind footprint, which is used as an index of body size. There is a clear positive association between these two variables, indicating that the home range sizes of males increase as they get larger or more mature. The home ranges of these males were very variable, but all large; the difficulty of attempting to sample from animals covering such areas of dense bushland is apparent, particularly when location of animals and subsequent tracking to obtain urine samples were largely opportunistic.

Table 1. Comparison of home range size and hind footprint width in male black rhinoceros.

Rhinoceros I.D.	Hind foot width (cm)	Home range size (km^2)
L	21.75	54
Z	21.5	31
U	21.0	31
X	20.5	25
M2	20.5	17
D	19.75	15

Males L and X were both known breeders, but did not have the largest home ranges. The levels of oestrone conjugates detected in the urine of these six males showed no relationship to the link between home range size and body size.

Assessment of reproductive status

Males

Testosterone

Because only small amounts of urine could be recovered from free-ranging males, initial attempts to assay urinary testosterone were confined to samples from captive animals. The results shown in Table 2 indicate that levels of total urinary testosterone (i.e. conjugated and unconjugated) in male black rhinoceros were extremely low. Levels, even in samples of 0.5–1.0 ml, often bordered on the limit of sensitivity of the assay, making accurate assessment difficult. Furthermore, the extensive overlap between values obtained in males and females clearly indicated the lack of association of this measurement with testicular function. Because of this lack of potential and the need for samples to be of large volume, the assay of samples from free-ranging males was not attempted.

Table 2. Total immunoreactive testosterone (ng/mg Cr) in urine samples from adult male and female captive animals. Mean ± s.e.m. (range).

Males	Females
0.85 ± 0.22 (0.11 – 2.6) $n = 12$	0.19 ± 0.07 (0.06 – 0.45) $n = 6$

Conjugated oestrone

Since large amounts of urinary oestrogens are excreted by stallions (Velle 1975), and male Indian rhinoceros (E. Wanjohi & J.K. Hodges, unpubl. data), it was reasoned that it might be possible to assess the reproductive status of male black rhinoceros by measuring conjugated oestrone instead of testosterone. Levels of urinary oestrone conjugates in urine samples from captive males and females and free-ranging male rhinoceros are shown in Table 3.

Table 3. Levels of oestrone conjugates (ng/mg creatinine) in urine from male and female captive and male free-ranging animals. Mean ± s.e.m. (range).

Captive		Free-ranging
Males	Females	males
4.74 ± 0.85 (0.82 – 16.1) $n = 27$	5.91 ± 1.5 (0.79 – 24.3) $n = 16$	2.68 ± 0.16 (0.76 – 6.1) $n = 41$

Conjugated oestrone was detectable in all of 41 urine samples collected from six free-ranging males. Levels were, however, highly variable within individuals (range in one captive male: 0.82–16.1 ng/mg Cr) and there was no difference between values in males and females during the oestrous cycle. Furthermore, there were no differences in levels of oestrone conjugates between free-ranging males, even though at least two males (L and X) were known breeders, and two other males (D and M2) were almost certain non-breeders. We can therefore exclude the measurement of oestrone conjugates in urine as having diagnostic value in identifying reproductive status in black rhinoceros.

In view of the lack of success in using the measurement of either testosterone or conjugated oestrone in assessing reproductive status in male rhinoceros, a more detailed analysis of urinary androgen metabolites was initiated. Preliminary gas chromatographic and mass spectrometric analysis of derivatized black rhinoceros urine collected from two captive males revealed the adrenal androgen androstane-triol-one as the most abundant metabolite present, whilst also detecting small but significant amounts of the testicular androgen androstanediol.

The lack of measurable quantities of testosterone was notable and may offer an explanation for our inability to establish a valid radioimmunoassay for the hormone. Since androstanediol is the major urinary metabolite of testosterone in man, its presence in rhinoceros urine is encouraging, and may also indicate a potential method for assessment of testicular function and breeding status in males of this species. The development of an immunoassay for measurement of androstanediol is currently under way.

Females

Pregnanediol-3-glucuronide

Since levels of urinary immunoreactive pregnanediol-3-glucuronide have previously been shown to increase during the later stages of pregnancy in captive black rhinoceros (Ramsay, Kasman & Lasley 1987; Hodges & Green in prep.), its measurement may also provide the basis for a pregnancy test for animals living in the wild.

Figure 3 presents data from pregnant and non-pregnant captive females and from 17 samples collected from free-ranging females in Laikipia. Very large differences between levels in late pregnant and non-pregnant captive animals are seen. Pregnanediol-3-glucuronide was detected in all field samples but in a range consistent with these animals being non-pregnant, or in early pregnancy. This assessment has been confirmed, as none of the females sampled has given birth in the 12 months since sampling.

As described in the methods section, the assay for pregnanediol is a simple micro-titre plate enzyme-based method (Hodges & Green in prep.). The several advantages of ELISA assays, including low cost, ease of performance

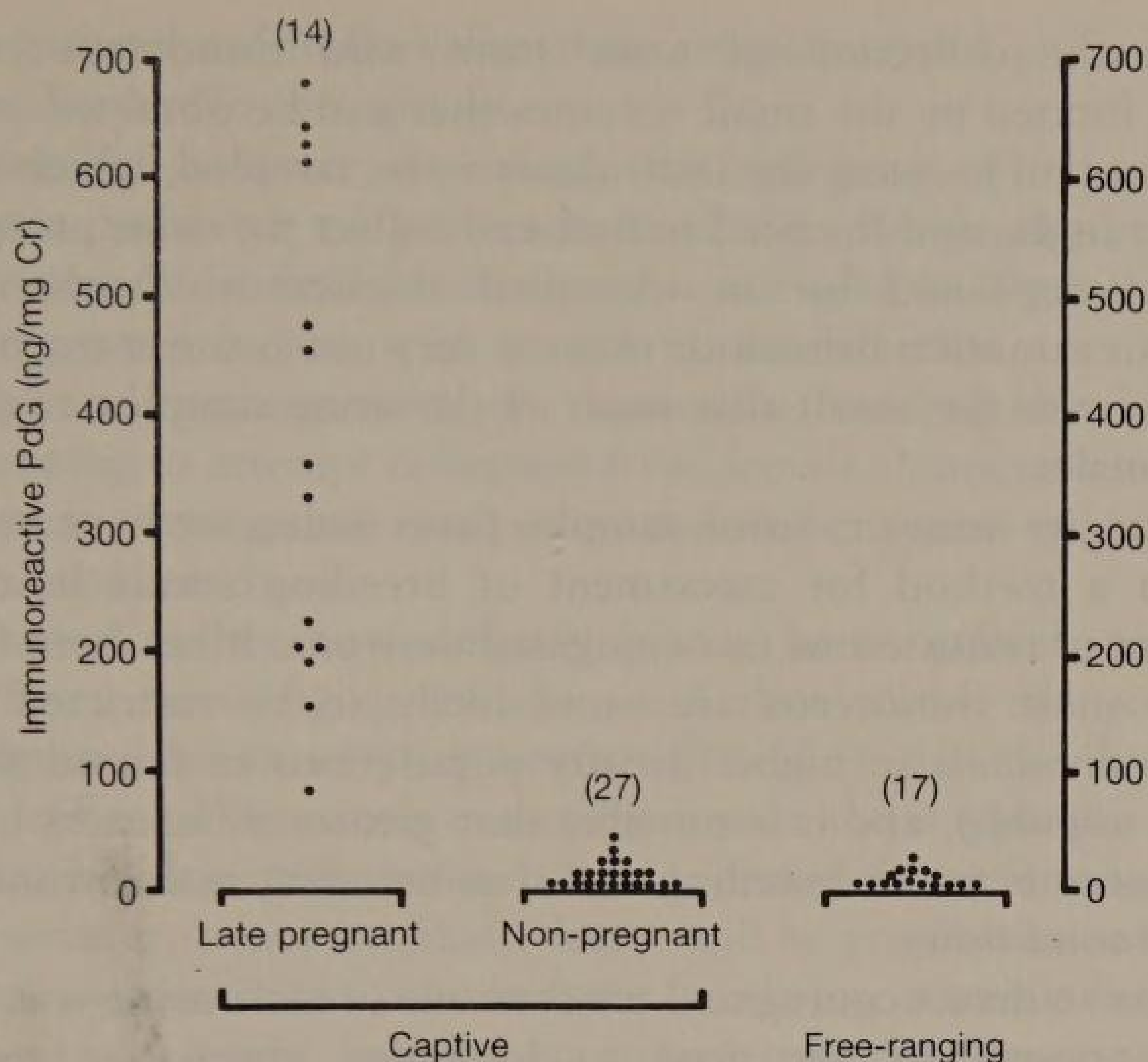

Fig. 3. Pregnanediol-3-glucuronide immunoreactivity (ng/mg Cr) in urine from pregnant and non-pregnant captive and free-ranging black rhinoceros. Numbers of samples in brackets.

and stability of reagents, are not only useful in a laboratory setting but also make them amenable to development for use under field conditions. In particular, the end point of the assay (i.e. colour change) is far simpler to quantify than the measurement of radioactivity, and consequently can be read without the need for sophisticated laboratory equipment. In order to assess the potential of the pregnanediol-3-glucuronide ELISA for field use we have evaluated a portable plate reader suitable for use under field conditions, by comparing the results obtained by this and a much larger standard laboratory machine.

A comparison of the results obtained with both plate readers showed a high degree of correlation over the maximum range of pregnanediol-3-glucuronide values normally found. The results gave a regression equation of $y = 1.14\,x - 0.03$ (where y = values were obtained by the portable plate reader, and x = values were obtained by the laboratory reader) and a correlation coefficient $r = 0.98$. The data shown in Fig. 3 were obtained using the portable plate reader.

Discussion

This paper establishes for the first time the feasibility of collection of urine from black rhinoceros in the field and the potential usefulness of urinary hormone analysis in monitoring reproductive status.

Although the collection of urine from wild rhinoceros is feasible, sampling is limited by the small volumes that can be obtained (< 1.0 ml), the uncertainty of locating the individuals to be sampled, which all occupy large home ranges, and the need to find and collect the urine promptly after it has been deposited by an identified rhinoceros. Furthermore, the differences in urination behaviour make it very much easier to collect urine from males, with the result that most of the urine samples analysed here were from males.

Despite greater access to urine samples from males, we have been unable to establish a method for assessment of breeding status based on the measurement of testosterone or conjugated oestrone. It has been found that matings by male rhinoceros are more likely to be restricted to a few dominant individuals in higher-density populations in fenced sanctuaries (R.A. Brett unpubl.), and it is possible that greater differences in levels of these hormones between breeding and non-breeding males would be seen under these conditions.

The failure to detect conjugated testosterone in male urine was surprising but clearly points to the need to look for alternative metabolites. Androstanediol is one possibility which we are currently investigating further. Thus, although the identification of the major urinary metabolite of testosterone may eventually lead to successful physiological assessment of male breeding status, for the present such assessment must chiefly rely on observation of matings by known males, and of regular consorting by particular males with females which are likely to be in oestrus.

It was found that larger and probably more mature males had larger home ranges. It remains to be determined whether these larger males are necessarily breeders, or occupy a minimum home range size that may be used as another indicator of breeding status. If a correlate of breeding status in the form of a urinary androgen is found, it should be possible to determine which males are breeding, simply from its levels in urine. However, given recent information about the earless male rhinoceros in Addo Elephant National Park that was castrated yet continued to guard females and attempt to mate (Hall-Martin 1986), this is open to doubt. Hall-Martin's observations should be taken into account by managers of rhinoceros sanctuaries: whilst castration might prevent further genetic contributions from males that have sired too many calves in a given sanctuary, it might lead to behavioural problems and obstruction from the castrates.

Results on the assessment of reproductive status in females were more encouraging. Studies on captive female black rhinoceros indicate a variable but significant increase in the levels of immunoreactive pregnanediol-3-glucuronide during mid to late pregnancy (Ramsay *et al.* 1987; Hodges & Green in prep.), and the present data substantiate this. Given the variability in levels between animals and the limited number of data available, it is

difficult to gauge the ultimate value of the pregnanediol-3-glucuronide assay as a pregnancy test in this species.

At the moment, however, we can say that high levels of immunoreactivity are a certain indicator of pregnancy and that the method required to perform the test under field conditions is now available. The value of such a test for pregnancy in the wild would be considerable as a tool in the monitoring and management of rhinoceros sanctuaries, and we are currently hoping to attempt collection from female rhinoceros in more open habitat, where matings are easily recorded and progress of gestation and parturition monitored.

It was noted earlier how difficult it may be to collect urine samples regularly from free-ranging females, and thus allow the possibility of accurate determination of peak oestrus. The fact that urine of oestrous females is deposited frequently in small amounts and is typically not sprayed means that, besides urine being hard to obtain from female rhinoceros outside oestrus, it is most unlikely that it will be possible to collect regular urine samples of sufficient volume from free-living oestrous females.

This should be borne in mind in considering the future possibility of artificial insemination of female black rhinoceros in sanctuaries. Except by observing matings by resident males, it may prove impractical to monitor oestrus accurately in any female black rhinoceros except those in captive conditions. It should also be noted that levels of oestrogens in the urine of captive female black rhinoceros so far show no relationship to the onset or termination of oestrus (Ramsay *et al.* 1987), and at present it is not possible to detect the time of ovulation in this species using any method of urinary hormone analysis.

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