RECENT ADVANCES IN REPRODUCTIVE MONITORING OF RHINOS IN CAPTIVITY AND IN THE WILD

Joanne E. Hindle[1], J.Vahala[2] and J.K.Hodges[3]
[1]Institute of Zoology, Zoological Society of London, Regent's Park, London NW1 4RY, UK.
[2]Zoological Garden, Dvur Kralove n.L., Czechoslovakia
[3]Division of Reproductive Biology, Deutsches Primatenzentrum GmbH, Kellnerweg 4, D-3400 Gottingen, Germany.

Despite recommendations of the IUCN Captive Breeding Specialist Group (CBSG) to conduct reproductive studies on rhinos in captivity to gain insights into their reproductive biology and physiology, progress in the development of non-invasive methods for assessing reproductive status has been slow. In 1981, Kassam and Lasley (1981) identified oestrone sulphate as the major urinary oestrogen in the Indian rhino. The most abundant urinary progesterone metabolite was later identified by Hindle, *et al.*, (1988) as pregnanediol glucuronide (PdG), and the simultaneous measurement of these two steroid metabolites has provided valuable information on ovarian function and pregnancy (Kasman, *et al.*, 1986; Hodges and Green, 1989) in the Indian rhino. However, low or undetectable levels of PdG immunoreactivity were reported in the urine of African rhinos during oestrous cycles (Ramsay, *et al.*, 1987; Hodges and Hindle, 1988) and, although oestrone conjugates were detectable, the pattern of excretion showed no correlation with reproductive events.

Thus, information on the reproductive physiology of rhinos is extremely limited yet essential for their reproductive and genetic management both in captivity and in their native countries where populations are becoming increasingly fragmented. The aim of the present study was to identify and measure the most abundant oestrogen and progesterone metabolites in the urine of African rhinos with the specific objective of developing accurate and reliable methods for assessing reproductive status in these species.

ANIMALS AND SAMPLE COLLECTION

Hormonal profiles were compiled by analysing urine samples collected from seven adult female black rhinos (*Diceros bicornis*) housed and maintained by the Zoological Society of London, UK, St. Louis Zoo, USA, Dvur Kralove Zoo, Czechoslovakia, and Audubon Zoo, USA; one adult, female southern white rhino (*Ceratotherium simum simum*) housed and maintained by Paignton Zoo, UK, and two adult, female northern white rhinos (*Ceratotherium simum cottoni*) housed and maintained by Dvur Kralove Zoo. Urine samples were collected daily during oestrous cycles, whilst urine was obtained at weekly intervals during pregnancy. Except during pregnancy, females were allowed access to a male of the same species and animals were observed for signs of mating or behavioural oestrus, indicated by increased frequency of urine spraying, whistling or interest in the male. Urine was aspirated from the floor of the enclosure, with the minimum inclusion of extraneous material, and frozen at -20°C. Upon first thawing, all samples were analysed for creatinine concentration by the method of Hodges and Green (1989).

HORMONE ASSAYS

In order to measure 20α-dihydroprogesterone (20α-DHP) in hydrolysed rhino urine, a sensitive enzyme immunoassay (EIA) was developed using an antiserum raised against 4-pregnen-20α-ol-3-one carboxymethyloxime:BSA and an enzyme label of horse radish peroxidase conjugated to 20α-DHP (Hindle *et al.*, 1991). Standards (1600-6.4 pg/ml) and samples were incubated with enzyme label (1:30,000) and antiserum (1:150,000) in duplicate wells on a microtitration plate, pre-coated with sheep anti-rabbit IgG. Plates were emptied, washed and developed with an enzyme substrate of o-phenylenediamine. Absorbance was measured at 492 nm. Steroids showing significant cross reactivity, determined at 50%

binding, were 5ß-pregnan-20α-ol-3-one (22.0%), 5ß-pregnan-3α,20α-diol (9.3%), 5-pregnen-3ß-ol-20-one (6.4%), 5ß-pregnen-20α-ol-3-one (3.0%) and progesterone (2.4%). Serial dilutions of hydrolysed and extracted urine from black and white rhinos during the post-oestrus period gave displacement curves parallel to that obtained with the 20α-DHP standards. The sensitivity of the assay was 13 pg/ml; inter- and intra-assay coefficients of variation (CV) were 6.9% (n=21) and 7.4%, respectively.

PdG immunoreactivity was measured by the EIA previously described and validated for African rhinos by Hodges and Green (1989). The sensitivity of the assay was 10 ng/ml; inter- and intra-assay CV were 9.1% (n=29) and 9.4% respectively. Total oestrogen immunoreactivity was measured by the EIA of Mostl, *et al.*, (1987), which used an antiserum that cross-reacted with oestradiol-17ß (161%), oestradiol-17α (63%) and oestriol (7.2%) (at 50% inhibition of binding with oestrone standards). The sensitivity of the assay was 6.4 pg/ml; inter- and intra-assay CV were 8.6% (n=17) and 11.8% respectively.

Oestradiol-17ß was measured by radioimmunoassay (RIA) as described by Shaw, *et al.*, (1989). The sensitivity of the assay was 40 pg/ml; inter- and intra-assay CV were 9.4% (n=11) and 11.2% respectively. Oestrone was measured by the RIA of Hodges, *et al.*, (1983). The sensitivity of the assay was pg/ml range; inter- and intra-assay CV were 9.9% (n=10) and 10.7% respectively.

IDENTIFICATION OF STEROID METABOLITES IN THE URINE OF AFRICAN RHINOS

Two approaches were taken to identify the major urinary metabolites of ovarian steroids in African rhinos. Firstly, a metabolism study was conducted (Hindle and Hodges, 1990), which involved the injection of radiolabelled oestradiol-17ß and progesterone into an adult female southern white rhino housed at Whipsnade Wild Animal Park (Zoological Society of London, UK). All urine and faeces were collected over the following 4 days and the distribution and identity of radioactive metabolites determined. A significant finding of this study was the absence of radiolabelled PdG in the urine, indicating species differences in progesterone metabolism between Indian and white rhinos. The only conjugated progesterone metabolite in the urine was 20α-DHP. Exogenous oestradiol-17ß was excreted as oestrone and oestradiol-17ß, although significant amounts of oestradiol-17α were also found in the urine.

In view of these findings, the metabolism and excretion of endogenous steroids was investigated in both white and black rhinos using high performance liquid chromatography (HPLC) and sequential hydrolysis techniques as described by Hindle, *et al.*, (1991). The elution profile of 20α-DHP immunoreactivity following HPLC of hydrolysed urine collected from a female northern white rhino during the luteal phase (oestrus+7 days) of the ovarian cycle (Fig. 1a) showed a single peak of immunoreactivity co-eluting with [^{3}H]20α-DHP. Similar profiles were obtained for luteal samples from black rhinos. In addition, HPLC confirmed the absence of PdG in urine collected during the luteal phase (Fig. 1b), but indicated the presence of a contaminant which eluted at a constant retention time with respect to PdG in both species. HPLC of hydrolysed urine collected on the day prior to oestrus indicated a single peak of immunoreactivity co-eluting with [^{3}H]oestradiol-17ß in samples from northern white rhinos (n=8), whilst in the black species immunoreactivity was present in the fractions containing the oestrone marker (Fig. 2 a and b).

Sequential hydrolysis indicated that, of the total 20α-DHP immunoreactivity, most (85%) was conjugated; glucuronides predominated in post-oestrus urine from black rhinos whereas sulphates were more abundant in white rhinos (Table 1). Over 90% of urinary oestrogen was conjugated in both species, with oestradiol-17ß glucuronide predominating in the white rhino and oestrone glucuronide being most abundant in the black rhino. As over 85% of ovarian steroids were conjugated in the urine of African rhinos, all urine samples were hydrolysed with a non-specific ß-glucuronidase aryl-sulfatase and extracted with diethyl ether prior to assay for 20α-DHP, oestradiol-17ß and oestrone (Hindle, *et al.*, 1991).

STEROID EXCRETION DURING THE OVARIAN CYCLE

Profiles of urinary 20α-DHP and oestradiol-17ß through two oestrous cycles in a northern white rhinos (Fig. 3a) showed a cyclic pattern of excretion. Concentrations of 20α-DHP were low (<10 ng/mgCr) for at least 10 days prior to mating while levels of oestradiol-17ß rose to peak levels on the day of or the day preceeding mating. Mating was followed by a rapid increase in 20α-DHP excretion to reach peak levels (60-80 ng/mgCr) within 8-12 days, co-incident with the nadir in oestradiol-17ß excretion. Similar data were obtained from the southern white rhino (Fig. 3b) although oestrus was not detected. Levels of 20α-DHP were <10 ng/mgCr during the presumed follicular phase and rose to reach maximum concentrations (approximately 80 ng/mgCr) following peak oestradiol-17ß excretion.

A representative cyclic profile of 20α-DHP and oestrone excretion in a black rhino is shown in Fig. 4. Concentrations of 20α-DHP before oestrus were extremely low (<1 ng/mgCr) for a shorter period than in the white rhino, while oestrone levels rose to peak on the day of oestrus. Oestrus was followed by an immediate increase in 20α-DHP excretion to reach maximum concentrations (6-8 ng/mgCr), ten fold lower than in the white rhino. A composite profile of urinary 20α-DHP and oestrone excretion aligned to the day of oestrus/mating in 4 black rhinos (Fig. 5) indicated a follicular phase (20α-DHP levels below 1 ng/mgCr) of 3-4 days and a luteal phase of approximately 17 days. Mean oestrone rose above baseline values (1 ng/mgCr) 5 days before oestrus to reach maximum levels on the day of oestrus, and returned to baseline values within 5 days.

The time course of 20α-DHP and oestrogen excretion in relation to the occurrence of oestrus or mating suggests that the measurement of these metabolites closely reflects follicular development and luteal function. However, without plasma progesterone data or direct evidence that the cycles described here were ovulatory, correlation of the timing of hormonal changes with ovarian events is difficult. The length of the oestrous cycle may be estimated from the data as 21-22 days in the black rhino and 24 and 25 days in the northern and southern white rhinos respectively.

STEROID EXCRETION DURING PREGNANCY

In addition to monitoring ovarian function, the pattern of excretion of steroid hormone metabolites may afford an insight into the endocrinology of pregnancy and provide a method for pregnancy diagnosis in African rhinos. Figure 6 shows levels of urinary 20α-DHP and PdG through conception, gestation and the *post-partum* period in a black rhino. Within 3 months of conception, levels of 20α-DHP had fallen to baseline values (<1 ng/mgCr) whilst levels of immunoreactive PdG were elevated (40-60 mg/mgCr) above those seen during the ovarian cycle in this animal. HPLC of urine collected during this period demonstrated that this immunoreactivity was due to the presence of PdG. Whilst 20α–DHP concentrations remained low, PdG levels continued to rise throughout the gestation period to peak 2 months prior to birth. After this point PdG levels fell to <5 ng/mgCr on the day of parturition. From the profile of progesterone metabolite excretion it was possible to determine a gestation length of 494 days. Urinary 20α-DHP remained undetectable for 20 days *post-partum* when concentrations rose rapidly to luteal levels. An increase in urinary oestrone from <1 ng/mgCr to 5 ng/mgCr on day 20 *post-partum* suggested that ovulation had occurred. No behavioural oestrus was seen as the female was not housed with a male.

In contrast, data from a northern white rhino (not shown) indicated that PdG did not become the most abundant urinary progesterone metabolite until 7 months after conception. Levels of 20α-DHP remained elevated (20-160 ng/mgCr) throughout gestation until levels of both metabolites fell to baseline (<10 ng/mgCr) at parturition, where they remained constant until at least 3 months *post-partum*, suggesting the absence of a *post-partum* ovulation in this species.

The apparent shift in excretion of progesterone metabolites, from 20α-DHP to PdG should allow accurate pregnancy diagnosis in African rhinos. Although the time at which PdG becomes the major progesterone metabolite varies between species, the presence of elevated levels of PdG indicates pregnancy in captive African rhinos (Ramsay, *et al.*, 1987; Hodges and Hindle, 1988; Hodges and Green, 1989) and free-ranging black rhinos (Hindle, Brett and Hodges, unpub. obs.). Methods for pregnancy detection could be improved by the use of antisera specific for PdG or chromatographic separation of PdG prior to immunoassay, both of which would potentially allow pregnancy detection from a single urine sample.

In conclusion, we have provided valuable information on the excretion of steroid metabolites during the reproductive cycle of African rhinos. The previous lack of success in assessing reproductive status in African rhinos appears to be due to species differences in progesterone metabolism between Indian and African rhinos. Use of a sensitivie EIA for 20α-DHP has allowed the oestrous cycle of African rhinos to be monitored for the first time. An apparent shift in progesterone metabolite excretion during gestation provides the basis for methods for pregnancy detection in these species. The methods for monitoring reproductive function described here may now be applied to the management of natural breeding of African rhinos in captivity and to allow the development and application of reproductive techniques for artificial (assisted) breeding. In addition, methods for assessing reproductive status may also be applied to reproductive studies and genetic management of small populations in the wild.

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TABLE 1

	Fraction			
	Unconjugated	Glucuronide	Sulphate	Residual
Northern white rhinoceros				
20α-DHP[1]	9.5±1.5	23.6±4.3	56.9±4.5	13.9±2.7
Oestradiol-17β[2]	1.1±0.4	80.2±1.4	2.16±0.7	16.1±1.0
Black rhinoceros				
20α-DHP[3]	14.6±2.2	49.4±3.1	13.4±2.5	14.9±2.7
Oestrone[4]	1.4±0.3	77.8±2.5	17.3±1.2	3.5±1.0

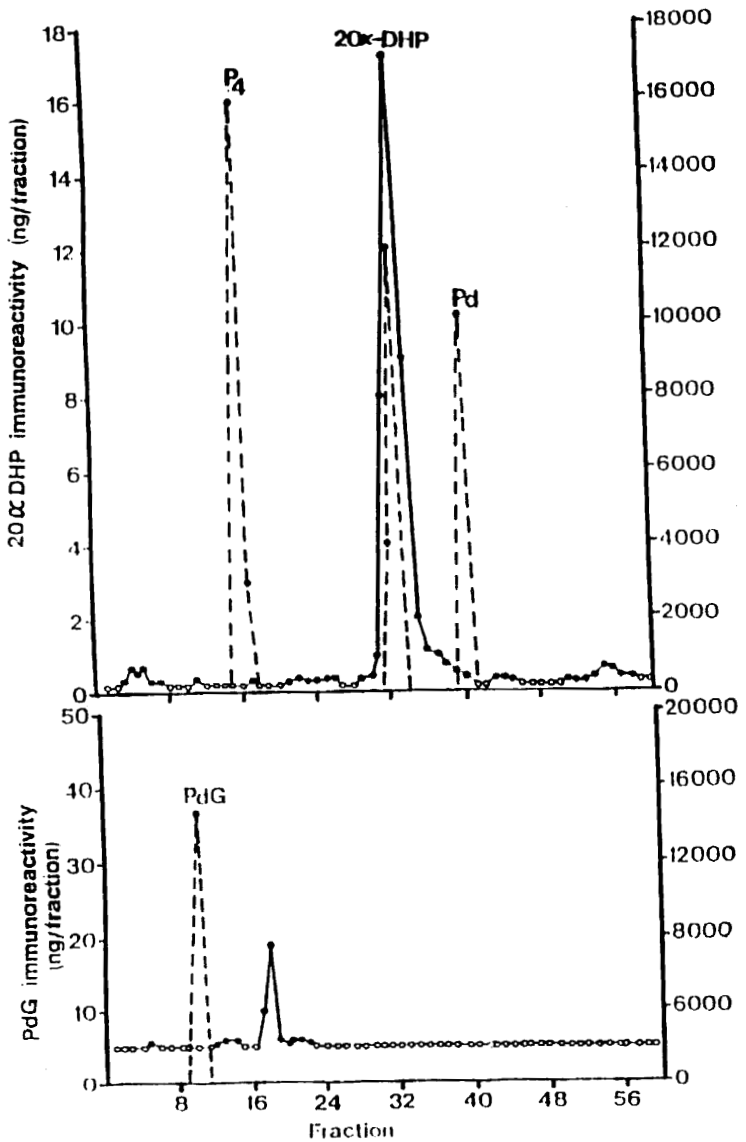

Figure 1. HPLC elution profiles of immunoreactive progesterone metabolites in urine collected from a northern white rhino 7 days after oestrus. In a, the solid line represents the elution profile of 20α-dihydroprogesterone immunoreactivity in hydrolysed urine, compared to that of ^{3}H-labelled progesterone (P_4), 20α-DHP and pregnanediol (Pd) (-----). In b, the elution profile of pregnanediol glucuronide (PdG) ( ) is shown along with that of the ^{3}H-labelled standard (----). Open circles represent levels of immunoreactivity below the sensitivity of the assay. Figure modified from Hindle *et al.*, 1991.

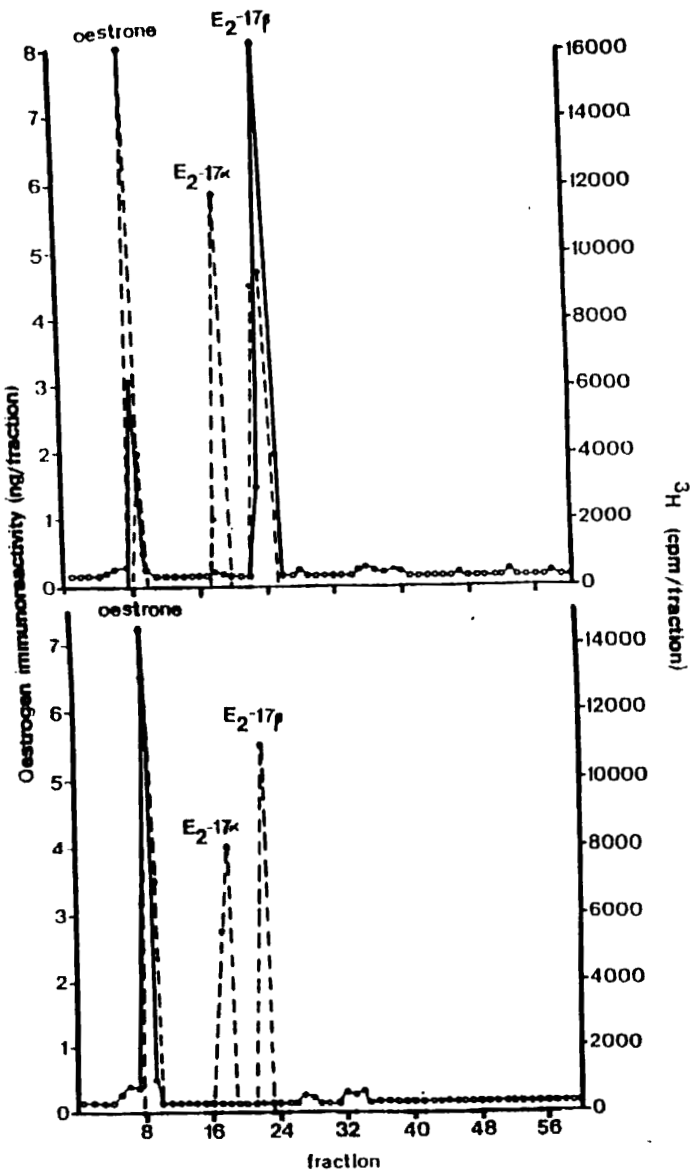

Figure 2. HPLC elution profile of ^{3}H-labelled oestrogens (----), oestrone (E_1), oestradiol-17α ($E_2$17α) and oestradiol-17β ($E_2$17β) and oestrogen immunoreactivity ( ) in hydrolysed urine from the northern white (a) and black (b) rhino on the day prior to oestrus. Figure taken from Hindle *et al.*, 1991, reproduced with permission from Journal of Reproduction and Fertility.

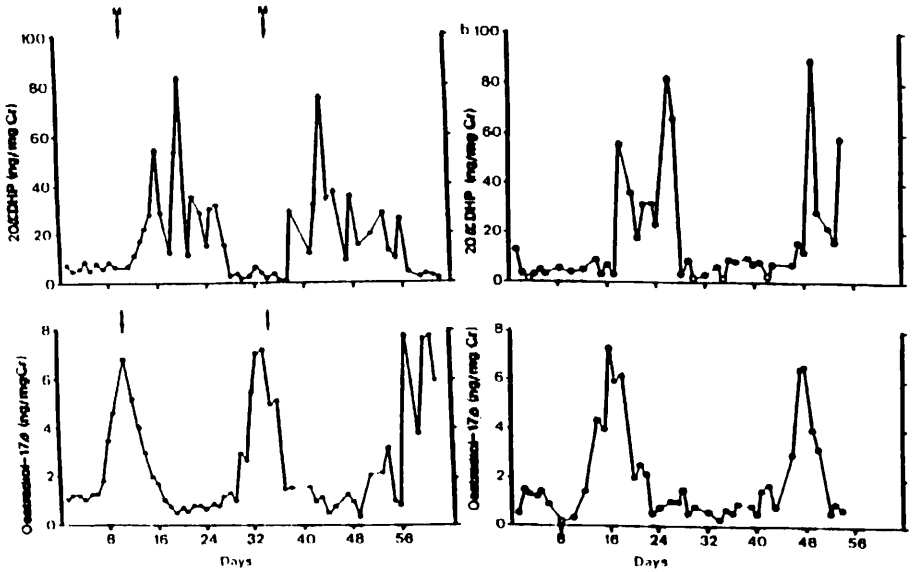

Figure 3. Pattern of excretion of 20α-dihydroprogesterone (20α-DHP) and oestradiol-17β during successive oestrous cycles in a northern (a) and southern (b) white rhino. Arrows indicate the time of mating (M). Open circles represent values that were below the assay sensitivity. Figure taken from Hindle *et al.*, 1991, reproduced with permission from Journal of Reproduction and Fertility.

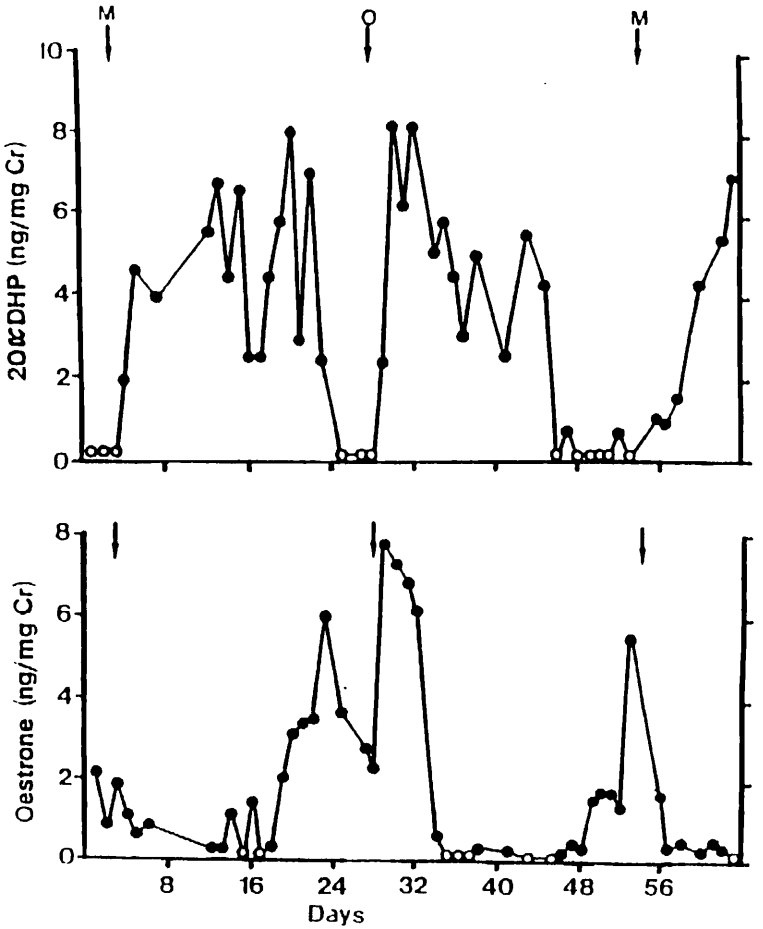

Figure 4. Pattern of excretion of 20α-dihydroprogesterone (20α-DHP) and oestrone during successive oestrous cycles in a black rhino. Arrows indicate the time of observed behavioural oestrus (O) or mating (M). Open circles represent values that were below the assay sensitivity. Figure modified from Hindle *et al.*, 1991.

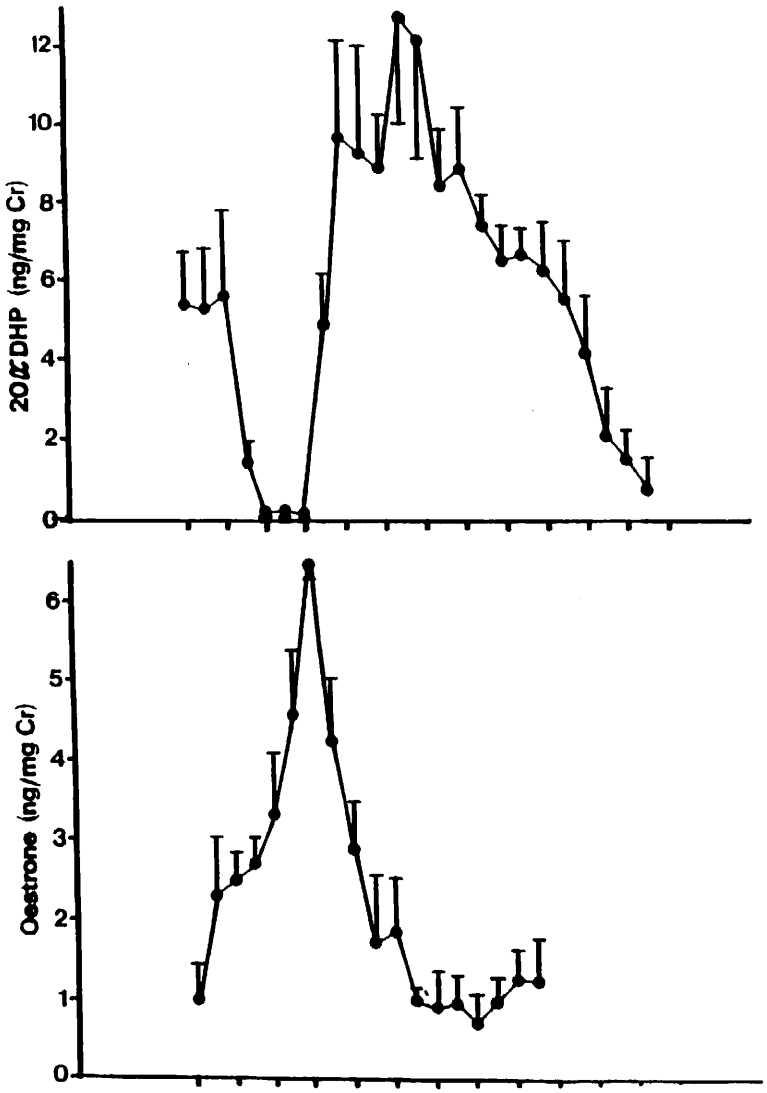

Figure 5. Mean±s.e.m. urinary 20α-dihydroprogesterone (20α-DHP; a) and oestrone (b) concentrations during 9 oestrous cycles in black rhinos (n=4). Individual cycles were standardized at Day 0, corresponding to the day of oestrus or mating. Each point is mean±s.e.m. of individual values obtained for a particular day (n=6-9). Figure taken from Hindle *et al.*, 1991, reproduced with permission from Journal of Reproduction and Fertility.

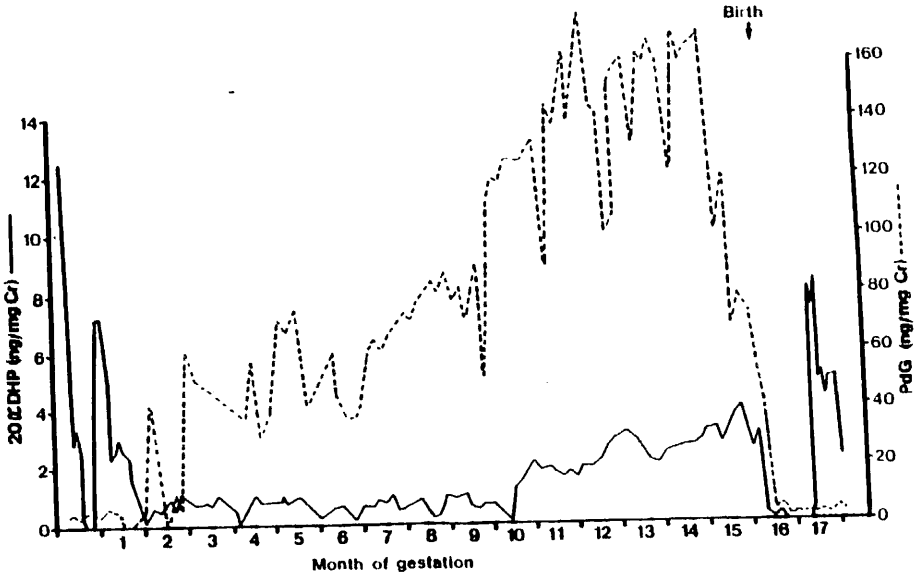

Figure 6. Profile of excretion of 20α-dihydroprogesterone (20α-DHP;) and pregnanediol glucuronide (PdG; ---) throughout a conception cycle, complete pregnancy and the *post-partum* period in a black rhino.