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Metabolism and excretion of oestradiol-17β and progesterone in the Sumatran rhinoceros (*Dicerorhinus sumatrensis*)

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Abstract

^{3}H-labelled oestradiol-17β and ^{14}C-progesterone were injected i.v. into an adult female Sumatran rhinoceros (*Dicerorhinus sumatrensis*) and all urine and faeces collected over 4 days. Of the injected steroid, 68% of ^{3}H-oestradiol and 89% of ^{14}C-progesterone were recovered. Peak excretion in urine occurred on day 1 for both steroids, and for faeces on day 2 for ^{14}C-progesterone, and between days 2 and 3 for ^{3}H-oestradiol. Oestradiol metabolites were predominantly (nearly 70%) excreted into the urine, while progesterone metabolites were almost exclusively (> 99%) excreted into the faeces. The majority (> 70%) of urinary excreted oestrogens consisted of water-soluble (i.e., conjugated) forms, with > 90% of these being glucuronides. In contrast, > 75% of faecal oestrogen and progesterone metabolites were excreted as ether-soluble (i.e., unconjugated) forms. HPLC co-chromatography of oestrogens in hydrolysed urine indicated only one peak of radioactivity, co-eluting with authentic oestradiol-17β, whereas two peaks of radioactivity were found after HPLC of faecal oestrogens, the major one co-eluting with oestrone and the less prominent one with oestradiol-17β. Progesterone was excreted as numerous metabolites into the faeces. The three most abundant of these were identified using HPLC and gas chromatography mass spectrometry (GCMS) as 5β-pregnane-3α,20α-diol, 5β-pregnane-3α-ol-20-one, and a second pregnanediol, the exact structure of which could not be deduced. Measurement of urinary oestradiol-17β and faecal immunoreactive pregnanediol and 5α-pregnane-3α-ol-20-one

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in daily samples enabled the first endocrine characterization of the ovarian cycle and indicated a cycle length of ~25 days. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Sumatran rhinoceros; Steroid metabolism; Urinary and faecal hormone excretion; Ovarian cycle; Non-invasive monitoring

1. Introduction

Although all rhinoceros species are threatened in the wild, the situation for the Sumatran rhinoceros (*Dicerorhinus sumatrensis*) is probably the most critical. Due to continued poaching and extensive destruction of its native habitat, the number of wild animals has declined to < 400 individuals which now live in small, highly fragmented populations in Southeast Asia. Only Indonesia and Malaysia are considered significant range states (http://www.rhinos-irf.org/programs/sumatranprograms.html). As the decline in current population continues at a rapid rate (half of the population has been lost in the last decade), the species is at high risk of extinction and is listed on Appendix I of CITES.

Apart from strengthening habitat protection and antipoaching measures, considerable effort has been made to promote breeding of the Sumatran rhinoceros in captivity to conserve the species. However, no offspring have been produced in captivity and of the 20 animals currently held in zoos, none is actually breeding (http://www.rhinos-irf.org/programs/sumatranprograms.html). In addition to the problem of limited animal numbers and inappropriate housing conditions, the general lack of understanding of the reproductive biology of the species may be contributing to breeding failure. The inability to assess female reproductive status and in particular to reliably detect oestrus and/or ovulation has been a major practical limitation. Because Sumatran rhinoceroses are solitary in nature and tolerate conspecifics only during oestrus, appropriate timing of pairing is essential for breeding captive individuals. Without this ability, attempted pairings have resulted in violent interactions between the sexes, and lead to serious injuries and on one occasion death (Foose, 1997). In addition to supporting natural breeding, information on reproductive status is also necessary if assisted reproduction techniques are to be useful for future species management.

Reproductive monitoring of exotic animal species is best accomplished by measuring oestrogen and progesterone metabolites in urine and faeces collected non-invasively (Lasley and Kirkpatrick, 1991; Heistermann et al., 1995; Schwarzenberger et al., 1996a). Urinary and, more recently, faecal steroid analyses have provided valuable basic information on the endocrine characteristics of the ovarian cycle and pregnancy in the Indian rhinoceros (*Rhinoceros unicornis*) (Kassam and Lasley, 1981; Kasman et al., 1986; Hodges and Green, 1989), as well as the African black (*Diceros bicornis*) (Ramsay et al., 1987; Hindle et al., 1992; Schwarzenberger et al., 1993, 1996b) and white (*Ceratotherium simum*) (Hindle et al., 1992) rhinoceros. These studies have revealed considerable species differences in steroid metabolism and urinary hormone excretion within the *Rhinocerotidae*. For example, oestrone sulfate and oestrone glucuronide are the major oestrogens in the urine of the Indian (Kassam and Lasley, 1981)

and black (Hindle et al., 1992) rhinoceros, respectively, in contrast to the white rhinoceros which predominantly excretes oestradiol glucuronide (Hindle et al., 1992). Furthermore, whereas pregnanediol glucuronide has been identified as the most abundant urinary progesterone metabolite in the Indian rhinoceros (Kasman et al., 1986; Hindle et al., 1992), the white (Hindle and Hodges, 1990) and black (Hindle et al., 1992) rhinoceros mainly excretes 20α-dihydroprogesterone into urine. The white rhinoceros also excretes a significant proportion of oestradiol and progesterone metabolites into the faeces (Hindle and Hodges, 1990), and faecal progestin measurements have been used to monitor luteal function and pregnancy in both African rhinoceros species (Schwarzenberger et al., 1993, 1996b; Schwarzenberger and Walzer, 1995).

Information on the identity and route of excretion of oestradiol and progesterone metabolites for the Sumatran rhinoceros does not exist. Recent attempts to monitor ovarian function in captive animals using urinary assays have yielded confusing results (J.E. Hindle, personal communication; Heistermann, unpublished), indicating the need for more basic studies on steroid metabolism in this species. To this end, the aims of the present study were to: (1) determine the time course and distribution of ^{3}H-labelled oestradiol-17β and ^{14}C-labelled progesterone metabolites in urine and faeces of the Sumatran rhinoceros; (2) provide information on the identity of the major urinary and faecal metabolites of both hormones; and (3) assess the feasibility of measuring excreted oestrogens and progestins for monitoring the ovarian cycle.

2. Materials and methods

2.1. Animal and housing

The animal used in this study was an adult, female Sumatran rhinoceros (~15 years of age) maintained at Taman Safari, Bogor, Indonesia. During the experiment, the female was housed in an indoor enclosure and was separated from its male partner. Regular cyclic changes in sexual behaviour (male–female interest, female whistling behaviour) and cyclic changes in vulval appearance (swelling and coloration) suggested that the female was reproductively functional, although successful matings had not occurred.

2.2. Preparation and injection of radiolabelled steroids

An infusion mixture containing 50 μCi ^{14}C-progesterone (48.9 mCi/mmol; NEN Du Pont, Dreieich, Germany) and 100 μCi ^{3}H-oestradiol-17β (40 mCi/mmol; NEN Du Pont) together with 100 μg oestradiol and 1000 μg progesterone was prepared in 1.6 ml ethanol. Sterile propylene glycol (3.4 ml, 30% in water, v/v) was added to the radiolabelled solution and the total volume injected into an ear vein. The animal was mildly sedated but still standing and feeding during the procedure. According to prior observations of sexual behaviour (see above), the female was assumed to be in the late luteal phase of the oestrous cycle. After isotope administration, the syringe and tube containing the radiolabel solution were each rinsed two times with 5 ml scintillation

fluid (Quickszint 2000; Zinser Analytic, Frankfurt, Germany), the residual radioactivity counted and subtracted from the pre-injection total to give the amount of radioactivity administered. All radioactive counting was conducted in 20 ml scintillation fluid by running a dual $^{3}H/^{14}C$ quench compensation program on a Packard TriCarb CA 2000 liquid scintillation counter.

2.3. Collection and storage of samples

Following radiolabel injection, all excreted urine and faeces were collected separately each day for 4 days (Days 1–4). To prevent cross-contamination, faecal material was removed within 5 min of voiding. The total amount of faeces excreted during each day was recorded, and two well-mixed aliquots (each 0.5 kg) were stored at −20°C until analysis. Urine was collected into 50 l plastic bags placed beneath the external outlet of the enclosure drain. The total volume of urine of each day was recorded and two well-mixed portions of 0.5 l were stored frozen with 0.1% sodium azide added as a preservative.

For monitoring ovarian activity, urine and faecal samples were collected daily between 0800 and 1000 h for a 2-month period. Observations on sexual behaviours (male–female interest, female whistling) were conducted in parallel to determine behavioural oestrus.

2.4. Sample analysis

2.4.1. Distribution of radioactivity

Radioactivity in duplicate 1 ml aliquots of freshly thawed urine from each day of sampling was determined by counting for 30 min. Faecal samples (duplicates) were analysed by subjecting a well-mixed portion of 0.5 g wet material from each day to catalytic combustion in an oxygen stream according to the method of Peterson (1969). Following combustion of the faecal material, the resulting $^{3}H_2O$ and $^{14}CO_2$ were selectively absorbed in scintillation fluids and then directly counted for 30 min to determine radioactivity. Values were multiplied by urine volumes or faecal weights to determine total excreted radioactivity.

2.4.2. Separation of conjugated and unconjugated steroids

All subsequent analyses were carried out on samples collected on the days of peak radioactivity excretion (i.e. day 1 for urine and day 2 for faeces). The proportion of steroid metabolites excreted in urine as free or conjugated forms was assessed by ether–water extraction. Duplicate 2 ml aliquots were extracted with 15 volumes of diethylether by vortexing for 15 min. The aqueous phase was frozen, the ether decanted, evaporated to dryness and reconstituted in absolute ethanol. Radioactivity was determined in duplicate 50 μl aliquots (free fraction). The residual aqueous phase was subjected to sequential enzyme hydrolysis by incubation with 2500 U specific β-glucuronidase (without sulfatase activity, No. G 7396; Sigma Chemie, Deisenhofen, Germany) in 0.5 M NaAc Buffer (pH 6.8) overnight at 37°C. Liberated steroids were then extracted with 10 volumes diethylether, the ether evaporated, redissolved in ethanol

and duplicate aliquots (100 μl) measured for radioactivity (glucuronide fraction). The aqueous residual was adjusted to pH 4.7 before further hydrolysis with 2500 U β-glucuronidase/sulfatase (No. G 1512; Sigma Chemie) overnight at 37°C. Following ether extraction, the radioactivity was counted in duplicate 100 μl aliquots of the reconstituted extract (sulfate fraction).

The proportion of water- and ether-soluble steroid metabolites in faeces was determined in quadruplicate aliquots of 0.4 g lyophilised and pulverised faecal powder. Samples were homogenised in 3 ml water and extracted four times with 10 ml diethylether by vortexing for 20 min. Following centrifugation (2500 rpm; 5 min), the ether phases (free steroid fraction) of each extraction step were decanted. The remaining faecal pellets were dried and subjected to catalytic oxygen combustion as described above and the amount of radioactivity counted to determine the proportion of conjugates in the faecal sample. As the majority of metabolites of both steroids in faeces were ether-extractable (see Section 3), further analysis of the conjugate forms was not carried out.

2.4.3. HPLC co-chromatography

^{3}H-oestradiol metabolites were separated on a reverse-phase NovaPak C-18 column (3.9 × 150 mm, Millipore, Milford, MA, USA) using an isocratic solvent system of acetonitrile (ACN)/water (H_2O) (30/70, v/v) at a flow rate of 1 ml/min (Heistermann et al., 1993). Prior to HPLC, urine samples were ether-extracted to remove free steroids and the aqueous phase hydrolysed with 2500 U β-glucuronidase/sulfatase as described above. The subsequent ether extract was reconstituted in ACN/H_2O (50/50, v/v), 100 μl injected onto the HPLC and 30 fractions (1 ml each) collected. Each fraction was reduced in volume to ∼0.2 ml and the total volume counted for 30 min. For separation of oestrogens from faeces, samples were lyophilised and pulverised and 1.5 g of faecal powder was extracted three times with 15 ml absolute methanol. Extracts were pooled and loaded onto a SepPak C-18 column according to the method described by Palme et al. (1997). In brief, 2.5 volumes of sodium acetate buffer (0.2 M, pH 4.7) were added to the extract and the total volume passed through the SepPak column. Steroids were eluted with 2 × 5 ml dichloromethane, which was then evaporated, the residual was redissolved in 2-propanol and centrifuged at 4000 rpm for 10 min. The supernatant was evaporated to dryness, reconstituted in ACN/H_2O (50/50, v/v) and 100 μl were separated on HPLC as described above to generate the profile of ^{3}H-radioactivity.

The ^{14}C-progesterone metabolites in faeces were separated on a reverse-phase NovaPak C-18 column (3.9 × 300 mm, Millipore, Milford, MA, USA) with ACN/H_2O (40/60, v/v) as eluent at a flow rate of 1 ml/min (Heistermann et al., 1993). From each of the 140 fractions collected, 850 μl were reduced in volume to ∼0.2 ml and counted to generate the profile of ^{14}C-radioactivity. The remaining 150 μl of the fractions were evaporated and reconstituted in assay buffer to determine profiles of pregnanediol and 5α-pregnane-3α-ol-20-one immunoreactivity.

The HPLC analysis was also carried out to determine the specificity of both faecal progestin measurements from samples collected during the mid-luteal phase of the ovarian cycle. Faecal samples (0.1 g lyophilised powder, $n = 2$) were extracted twice

with 1.5 ml absolute methanol, which was evaporated to dryness, reconstituted in ACN/H_2O (50/50, v/v) and subjected to the same HPLC system as described above for the separation of radioactive progestins. For all HPLC runs, the presence of radioactive or immunoreactive peaks was compared to the elution positions of authentic ^{3}H-oestrogen and progestin tracers determined in separate runs immediately prior to each sample analysis.

2.4.4. Gas chromatography mass spectrometry (GCMS)

The GCMS analysis was employed to identify the steroids present in the major peaks of radioactivity in faeces and the principal immunoreactive faecal progestin detected in the 5α-pregnane-3α-ol-20-one assay. Faecal samples were subjected to HPLC as described above and fractions corresponding to the peaks from three HPLC runs were collected, pooled and evaporated to dryness. The final samples were reconstituted in 50 μl toluene of which 1 μl was subjected to GCMS analysis under conditions described previously (Hodges et al., 1994). Mass spectrometry profiles were scanned and identification of unknown peaks achieved by GC retention times, computer MS library search (Finnigan MAT Wiley Library, V5.0) and comparison of fragmentation patterns and retention times with those of steroid reference standards.

2.4.5. Hormone assays

Urine samples were centrifuged and a 100 μl aliquot was hydrolysed with 2500 U β-glucuronidase/sulfatase as described above. Following hydrolysis, samples were extracted with 10 volumes diethylether and reconstituted in 400 μl absolute ethanol. Combined hydrolysis and extraction efficiency, determined by the recovery of ^{3}H-oestrone-3-glucuronide (10 000 cpm) added to each sample prior to hydrolysis was $87.0 \pm 8.7\%$.

Oestradiol immunoreactivity was determined in a microtiterplate enzymeimmunoassay directly from the methanol extract after appropriate dilution in assay buffer. The assay utilizes an antiserum against oestradiol-6-CMO-BSA (Ab No. 1001, Biocline, Cardiff, UK) together with oestradiol-alkaline phosphatase (made in our laboratory) as label and oestradiol-17β as the standard. Relative to oestradiol-17β (100%), the antiserum showed a significant cross-reactivity only with oestrone (2.0%). For determination of oestradiol, duplicate 50 μl aliquots of diluted sample or standard (range 0.49–125 pg) were combined with label (50 μl) and antiserum (50 μl) and incubated overnight at 4°C. After incubation, the plates were washed four times, blotted dry and 150 μl of phosphatase substrate (Sigma No. 104, 20 mg in 15 ml substrate buffer) was added to each well. The plates were incubated for a further 2–3 h before absorbance was measured at 405 nm. Assay sensitivity at 90% binding was 1 pg. Intra- and interassay coefficients of variation were 4.6% and 6.9%, respectively. Serial dilutions of samples from the follicular and luteal phase of the ovarian cycle gave displacement curves parallel to that of the oestradiol standard.

To control for variations in the volume and concentration of the voided urine, the creatinine concentration of each sample was determined by a creatinine analyser (Beckmann Instruments Brea, CA, USA) using 1:10 diluted urine samples. Urinary

hormone concentrations are expressed as nanogram per milligram (ng/mg) creatinine (Cr).

Faecal samples were lyophilised, pulverized, and ~0.1 g of faecal powder was extracted two times with 1.5 ml absolute methanol by vortexing for 30 min. Following centrifugation, the supernatants were pooled and after dilution in assay buffer were assayed directly. Mean extraction efficiency, determined by the recovery of ^{3}H-progesterone (20 000 cpm) added to each sample prior to extraction, was $81.5 \pm 5.9\%$. Faecal methanol extracts were assayed for immunoreactive 5β-pregnane-3α,20α-diol (pregnanediol, Pd) and 5α-pregnane-3α-ol-20-one (5-P-3OH) in enzymeimmunoassays previously characterized by Heistermann et al. (1993) and Hodges et al. (1997), respectively. Assay sensitivities at 90% binding were 40 pg and 15 pg per well for Pd and 5-P-3OH, respectively. Intra- and interassay coefficients of variation were 6.8% and 10.7% for Pd measurements, and 8.5 and 12.1% for 5-P-3OH measurements. Samples from the follicular and luteal phases of the ovarian cycle produced displacement curves parallel to those of the respective standards in both assays.

3. Results

3.1. Radiometabolism study

The amounts of urine and faeces excreted over the 4-day radiometabolism study and the distribution of radioactivity are shown in Table 1. Total radioactivity recovered in urine and faeces was 68% for ^{3}H-oestradiol and 89% for ^{14}C-progesterone. Metabolites of ^{3}H-oestradiol were predominantly (67.8%) excreted into the urine, whereas ^{14}C-progesterone metabolites were almost exclusively (> 99%) eliminated via the faeces. Peak excretion of radioactivity in urine was found within the first 24 h following the radiolabel injection (Fig. 1), and > 95% of combined radioactivity had been excreted by

Table 1
Volume of urine and mass of feces excreted over the experimental period, and distribution of ^{3}H-oestradiol-17β and ^{14}C-progesterone radioactivity shown as a percentage of total administered

Day	Urine			Feces		
	Volume excreted (l)	Percentage of radioactivity		Mass excreted (kg)	Percentage of radioactivity	
		^{3}H	^{14}C		^{3}H	^{14}C
Day 1	15.4	38.7	0.36	16.1	3.1	11.9
Day 2	18.7	5.5	0.18	23.7	8.3	44.1
Day 3	28.4	1.6	n.d.	22.0	8.5	17.3
Day 4	15.0	0.34	n.d.	31.5	2.0	15.2
Total radioactivity recovered (%)		46.14	0.54		21.9	88.5

n.d. = Not detectable.

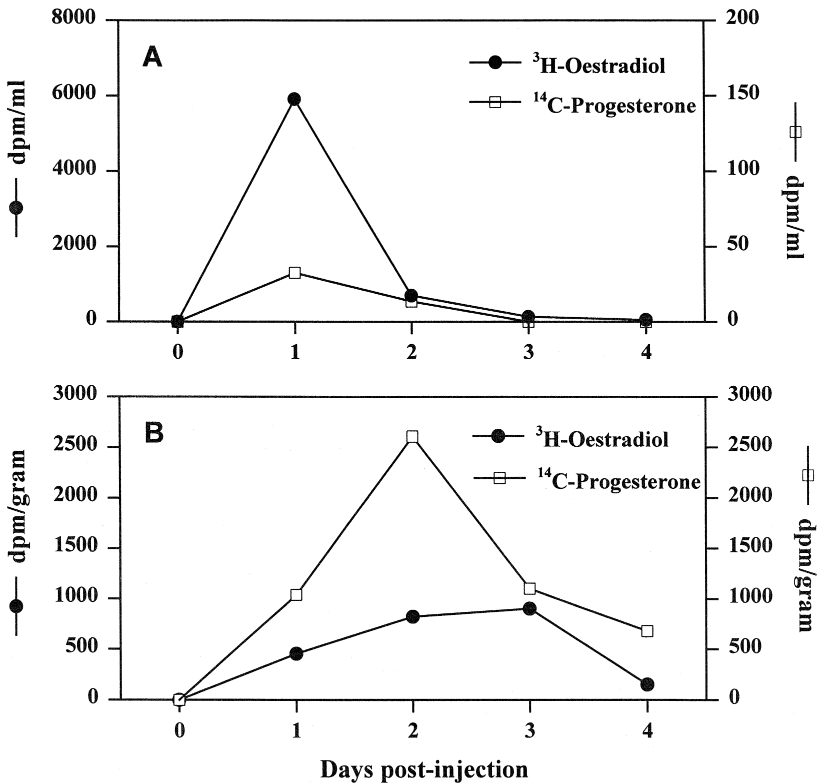

Fig. 1. Profiles of the excretory time course of ^{3}H-oestradiol-17β and ^{14}C-progesterone in (A) urine and (B) faeces after i.v. administration at day 0 in a female Sumatran rhinoceros.

the end of day 2. Peak excretion of radioactivity into faeces occurred on day 2 for ^{14}C-progesterone and between days 2 and 3 for ^{3}H-oestradiol (Fig. 1).

Table 2 shows that the majority (> 70%) of ^{3}H-radioactivity recovered from urine was associated with water-soluble (presumably conjugated) forms. In contrast, faecal metabolites of both steroids consisted primarily (> 75%) of ether-soluble (presumably

Table 2
Excretory fate of injected ^{3}H-estradiol-17β and ^{14}C-progesterone in the Sumatran rhinoceros

		Percentage of total radioactivity recovered[a]	Percentage of water-soluble (i.e., conjugated)[b]	Percentage of ether-soluble (i.e., unconjugated)[b]
Urine	^{3}H-estradiol	67.8	71.3	28.7
	^{14}C-progesterone	< 1	–	–
Feces	^{3}H-estradiol	32.2	24.6	75.4
	^{14}C-progesterone	> 99	20.2	79.8

Values represent the means from $^{a}n = 2$ or $^{b}n = 4$ determinations.

unconjugated) forms. Sequential enzyme hydrolysis of the conjugated portion revealed that almost 95% of urinary oestradiol metabolites were enzyme-hydrolysable, with ~ 90% of these conjugates being accounted for by glucuronides and ~ 10% by sulfates. HPLC co-chromatography of oestradiol metabolites in urine revealed a single major peak of radioactivity (fractions 14–16), that co-eluted with authentic oestradiol-17β standard (Fig. 2). In contrast, there were two distinct peaks of radioactivity in faeces, that co-eluted with authentic oestradiol-17β (28%) and oestrone (72%) (Fig. 2).

Isocratic HPLC of faecal ^{14}C-progesterone metabolites indicated four substantial peaks of radioactivity (Fig. 3a). Subsequent use of gradient HPLC (not shown), however, indicated that the radioactivity eluting in fractions 3–6 and 7–11 represented multiple small and quantitatively insignificant peaks, whereas the radioactivity in fractions 42–44 and 65–67 remained as single prominent peaks. The peak in fractions 42–44 co-eluted with authentic 5β-pregnane-3α, 20α-diol (pregnanediol, Pd) and application of a Pd immunoassay confirmed the presence of high levels of Pd immunoreactivity in the same fractions (Fig. 3b). The presence of large amounts of 5β-pregnane-3α, 20α-diol in fractions 42–44 was confirmed by GCMS, and although a second

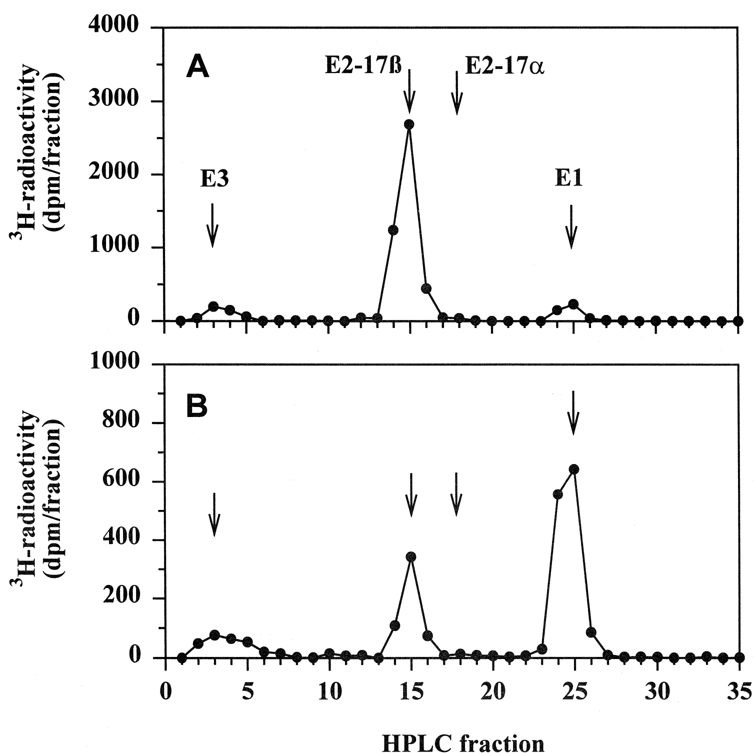

Fig. 2. The HPLC profiles of metabolised oestrogens in (A) urine and (B) faeces after i.v. injection of ^{3}H-oestradiol-17β in a female Sumatran rhinoceros. Retention times of radioactive peaks are compared to those authentic of oestriol (E3), oestradiol-17β (E2-17β), oestradiol-17α (E2-17α) and oestrone (E1) reference tracers.

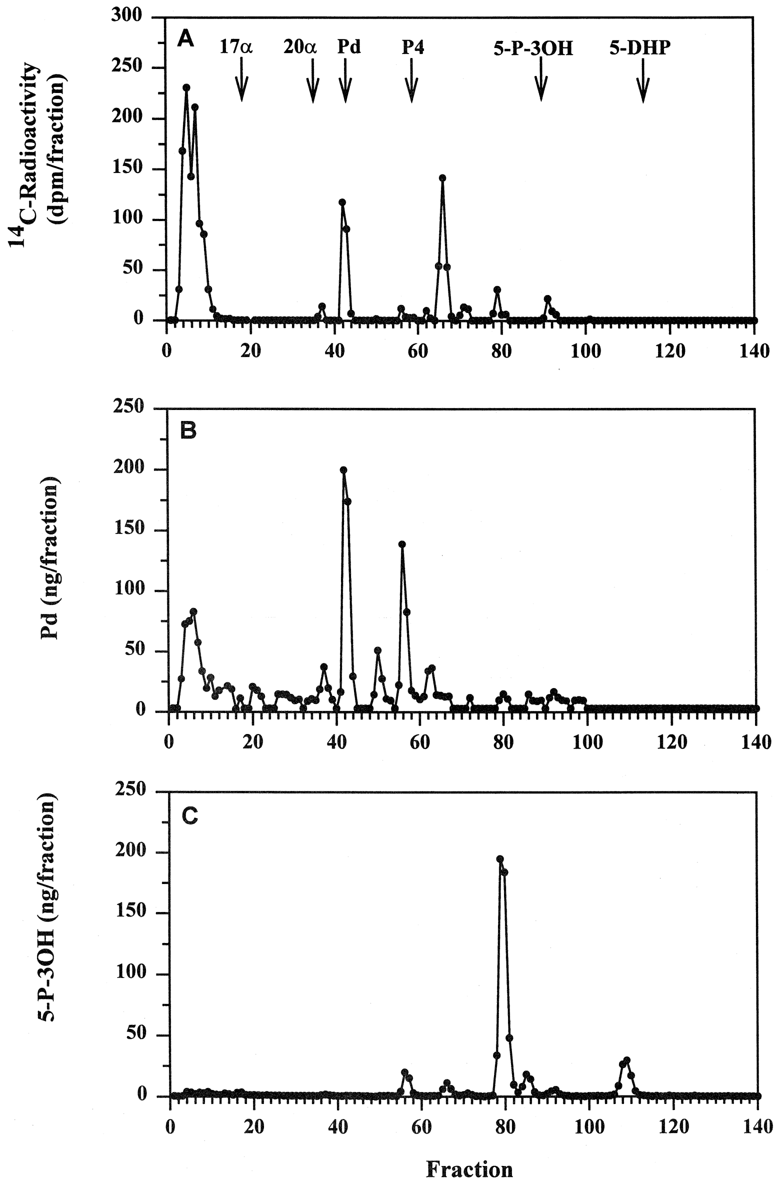

Fig. 3. The HPLC profiles of (A) ^{14}C-progesterone metabolites and (B) corresponding pregnanediol (Pd) and (C) 5α-pregnane-3α-ol-20-one immunoreactivity in the faecal sample of peak radioactive excretion. Retention times of ^{14}C-radioactive peaks are compared to those of authentic 17α-hydroxyprogesterone (17α), 20α-dihydroprogesterone (20α), 5β-pregnane-3α,20α-diol (Pd), progesterone (P4), 5α-pregnane-3α-ol-20-one (5-P-3OH) and 5α-dihydroprogesterone (5-DHP) reference tracers.

steroid with a molecular mass (320 kDa) and fragmentation pattern of a pregnanediol was also detected, in the absence of a complete range of reference standards, its exact structure could not be deduced.

The elution position of the radioactive peak in fractions 65–67 did not correspond to that of any of the progestin reference tracers tested. According to GCMS these fractions contained one major steroid with a molecular mass (318 kDa), fragmentation pattern and GC retention time identical to those of 5β-pregnane-3α-ol-20-one. A small peak of immunoreactivity corresponding to this major metabolite was detected in our 5-P-3OH assay (Fig. 3c), although cross-reactivity of this substance in the 5-P-3OH assay was < 9%. The major peak of immunoreactivity (fractions 78–81, Fig. 3c, insert in Fig. 4) was associated with a less polar steroid which eluted at the same position as a small peak of radioactivity as observed in Fig. 3a. According to GCMS, the identity of this steroid was 5α-pregnane-3β-ol-20-one which has a cross-reactivity of 295% in the

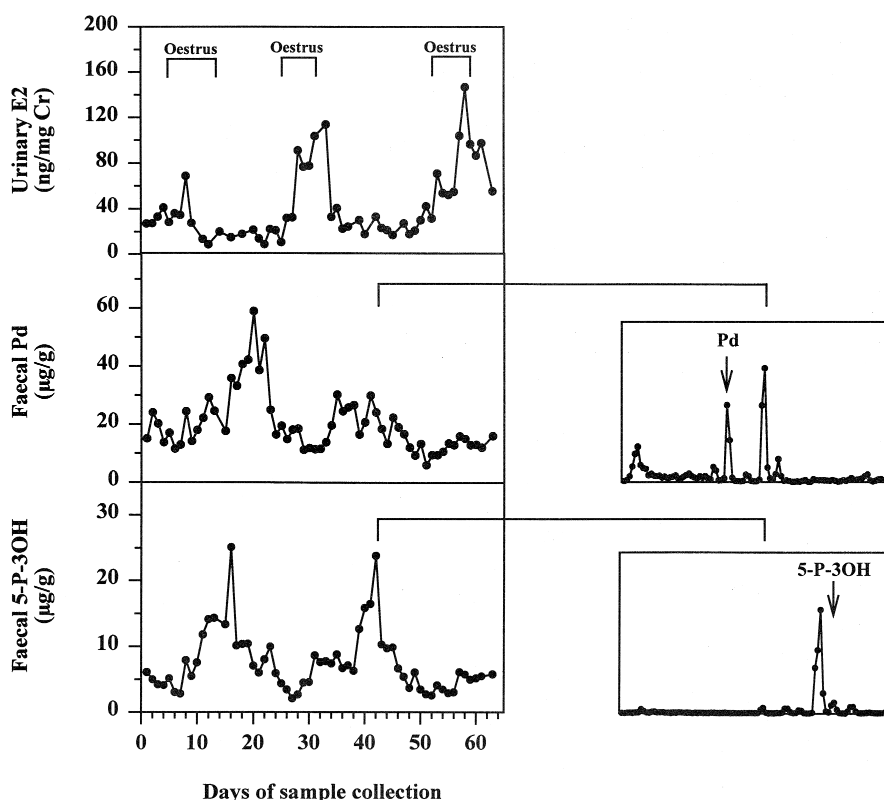

Fig. 4. Profiles of oestradiol-17β (E2) immunoreactivity in urine and pregnanediol (Pd) and 5α-pregnane-3α-ol-20-one (5-P-3OH) immunoreactivity in faeces are compared to periods of oestrus during two consecutive ovarian cycles in an individual female Sumatran rhinoceros. Inserts on the right show respective HPLC profiles of Pd and 5-P-3OH immunoreactivity obtained from a mid-luteal phase sample. Arrows indicate elution positions of authentic ^{3}H-Pd (Pd) and 5α-pregnane-3α-ol-20-one (5-P-3OH) tracers.

5-P-3OH assay. No significant peak of radioactivity was found at the elution position of authentic progesterone.

3.2. Oestrous cycle profiles

Based on the results of the radiometabolism study, urine and faecal samples collected from the same female over a 2-month period were analysed for immunoreactive oestradiol in hydrolysed urine, and Pd and 5-P-3OH immunoreactivity in methanol-extracted faeces (Fig. 4). Excretion of oestradiol immunoreactivity in urine showed a cyclic pattern with three distinct periods of elevated concentrations (75–150 mg/mg Cr), three to six times higher than baseline values (15–30 mg/mg Cr). In all cases, the periods of elevated oestradiol were associated with clear signs of behavioural oestrus as indicated by increases in whistling frequencies accompanied by a rise in proximity and interest between the male–female pair. Concentrations of immunoreactive faecal Pd were generally low (10–20 μg/g) before the oestradiol peak and increased (30–60 μg/g) during the presumed luteal phase. However, the luteal phase elevation in Pd levels was less clear in the second cycle as compared to the first. HPLC analysis of Pd immunoreactivity in luteal phase samples showed a similar profile as found in the radioactive sample, confirming that the measurement of Pd was non-specific (see insert, Fig. 4). The more specific measurement of faecal 5-P-3OH immunoreactivity revealed a clear cyclic pattern for both cycles with consistently low levels in the presumed follicular phase (3–8 μg/g) and three- to fivefold elevated concentrations (15–25 μg/g) in the presumed luteal phase. Based on the interval between the three successive oestradiol peaks, the length of each of the two cycles was 25 days, corresponding to a mean interval of 24 days between onsets of behavioural oestrus.

4. Discussion

This study provides the first information on the in vivo metabolism and excretion of exogenously administered radiolabelled oestradiol-17β and progesterone in a female Sumatran rhinoceros. A total of 68% of injected ^{3}H-oestradiol and 89% of ^{14}C-progesterone was recovered over the 4 days of sample collection, figures comparable to those obtained from radiometabolism studies in other species (e.g. Perez et al., 1988; Wasser et al., 1994; Brown et al., 1994, 1996; Palme et al., 1996), including the white rhinoceros (Hindle and Hodges, 1990). The 20% lower recovery for oestradiol metabolites compared to that for progesterone was most probably due to oestradiol being excreted primarily into the urine (in contrast to progesterone), some of which was absorbed into the substrate and did not flow into the outlet of the cage. Peak excretion of radioactivity into the urine occurred within the first 24 h, whereas highest amounts of radioactivity in faeces were found in samples from day 2 (progesterone) and between days 2 and 3 (oestradiol). More precise timing of peak excretion within these periods could not be determined as samples within each 24-h period were pooled before analysis. Nevertheless, the data are generally consistent with those reported for the white rhinoceros (Hindle and Hodges, 1990) and various other mammalian species (see Schwarzenberger et al., 1996a).

The present findings that oestradiol metabolites are excreted predominantly into urine, whereas progesterone metabolites are almost exclusively excreted into faeces are in marked contrast to those in the white rhinoceros, in which the majority of progesterone metabolites are excreted into urine while oestradiol is mainly eliminated via the bile into faeces (Hindle and Hodges, 1990). Although faeces represents the preferred route of steroid excretion in several other mammals (Schwarzenberger et al., 1996a), with the exception of carnivores, the virtually exclusive elimination of progestins into faeces as observed in the Sumatran rhinoceros is unusual. Although the reason for this is not clear, it would explain the low levels of progestins previously measured in urine and the lack of information on ovarian function provided by urinary progestin analysis in this species (Hindle, personal communication; Heistermann, unpublished). Significant amounts of progestins in faeces have nevertheless also been reported for the white and black rhinoceros in which their measurement was reliably used to monitor ovarian function and pregnancy (Schwarzenberger et al., 1993, 1996b; Schwarzenberger and Walzer, 1995).

The bulk of radioactivity in faeces of the Sumatran rhinoceros was found to be ether-extractable, presumably indicating steroids in an unconjugated form. In contrast, $> 70\%$ of the radioactivity in urine was associated with water-soluble, presumably conjugated metabolites. The excretion of predominantly conjugated steroids in urine and free hormones in faeces is similar to data for the majority of other species, including the white and black rhinoceros (Hindle and Hodges, 1990; Hindle et al., 1992; Schwarzenberger et al., 1996a). The finding that oestradiol-17β glucuronide is the only abundant oestrogen in the urine of the Sumatran rhinoceros is similar to that reported for the white rhinoceros (Hindle et al., 1992), but different from most other ungulate species, including the black (Hindle et al., 1992) and Indian (Kassam and Lasley, 1981; Kasman et al., 1986) rhinoceros, in which excretion of conjugated oestrone predominates. Free oestrone and oestradiol-17β accounted for virtually all oestrogens present in the faeces of the Sumatran rhinoceros, with oestrone being more abundant. Comparable information on the identity of faecal oestrogens in rhinos is only available for the white rhinoceros, in which oestrone was not found and both epimers of oestradiol accounted for all oestrogens detected (Hindle and Hodges, 1990).

The HPLC separation of faecal ^{14}C-progestins followed by GCMS analysis of the radioactive peaks revealed the presence of three abundant metabolites, but virtually no excretion of progesterone in its native form. In general, this finding is consistent with data in the black rhinoceros (Schwarzenberger et al., 1993, 1996b) and many other species (e.g. Heistermann et al., 1993, 1997; Shideler et al., 1993; Brown et al., 1994; Wasser et al., 1994; Palme et al., 1997) in which the metabolism of progesterone results in the excretion of a range of faecal progestins. But our results contrast with findings in the white rhinoceros, in which native progesterone was found to be the only significant faecal progestin (Hindle and Hodges, 1990). Specifically, the major faecal progesterone metabolites in the Sumatran rhinoceros were identified as two pregnanediols, one of which was 5β-pregnane-3α,20α-diol, and one 5-reduced 20-oxo pregnane, 5β-pregnane-3α-ol-20-one. Excretion of 5-reduced mono and/or dihydroxylated faecal pregnanes have also been reported for a number of other mammalian species (Brown et al., 1994; Wasser et al., 1994; Schwarzenberger et al., 1996a,b) and, in this respect, the

findings for the Sumatran rhinoceros compare well with these other studies. The presence of pregnanediols as abundant faecal progestins is, however, in contrast to findings in the white (Hindle and Hodges, 1990) and black (Schwarzenberger et al., 1993) rhinoceros in which these progesterone metabolites were either not present in the faeces or only found during pregnancy. With respect to progesterone metabolism, the Sumatran rhinoceros further differs from the black rhinoceros in that it predominantly excretes pregnanes of the 5β-series, whereas in the black rhinoceros only 5α-reduced pregnanes appear to be present in faeces (Schwarzenberger et al., 1996b). Moreover, the Sumatran rhinoceros does not appear to excrete 5α-dihydroprogesterone, which has been determined as an abundant faecal progestin in the black rhinoceros (Schwarzenberger et al., 1996b).

The data on endocrine cycle profiles presented in this study are preliminary and further studies are underway to confirm the present findings. Fig. 3 nevertheless shows the potential of urinary oestradiol measurements for monitoring follicular activity and moreover indicates that measurement of faecal immunoreactive pregnanediol and application of an assay against 5-reduced 20-oxo-pregnanes should be useful for assessing luteal function. Unfortunately, the pregnanelone assay used in the present study measures 5α-pregnane-3β-ol-20-one (5-P-3OH), a quantitatively minor progesterone metabolite, but did not significantly cross-react with 5β-pregnane-3α-ol-20-one, which was identified as one of the major faecal progestins (see above). Although the progestin profiles obtained with the non-specific Pd assay and the measurement of 5α-reduced 20-oxo pregnane immunoreactivity appear to be informative in reflecting luteal function, a specific measure of Pd or quantification of the 5β-reduced 20-oxo pregnane would likely result in a clearer profile.

Although oestradiol peaks were variable in both magnitude and duration, they were associated with behavioural oestrus and followed by immunoreactive progestin increases, providing indirect evidence that the profiles presented here reflect ovulatory cycles. The intervals between successive oestradiol peaks as well as those between periods of oestrus indicate a cycle length in this individual of ~25 days. This is similar to findings on cycle length in the black and white rhinoceros (Hindle et al., 1992; Schwarzenberger et al., 1993), but different from the ~45-day cycle for the more closely related Indian rhinoceros (Kassam and Lasley, 1981; Kasman et al., 1986). Given that no previous information on cycle length exists for the Sumatran rhinoceros, the data presented here need to be confirmed. The availability of reliable, non-invasive techniques to monitor ovarian function in this species (as described here) now makes this possible. Moreover, application of these methods will hopefully lead to a better understanding of the reproductive biology of the species and, in turn, improve the prospects for successful captive breeding.

5. Conclusions

(1) In the Sumatran rhinoceros, metabolites of oestradiol-17β are mainly excreted as conjugated forms into the urine, whereas those of progesterone are almost exclusively excreted as unconjugated forms into the faeces.

(2) Oestradiol-17β glucuronide is the only abundant oestrogen in urine, whereas oestrone is the major oestrogen in faeces, with oestradiol-17β being less abundant.

(3) Metabolism of progesterone is more complex and resulted in the excretion of three major metabolites, two pregnanediols, one of which was 5β-pregnane-3α,20α-diol, and a 5-reduced 20-oxo pregnane, 5β-pregnane-3α-ol-20-one.

(4) Measurement of oestradiol-17β in urine and pregnanediol and 5α-pregnane-3-ol-20-one immunoreactivity in faeces provides the first longitudinal profiles in this species and indicates an oestrous cycle length of ∼25 days.

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