Non-invasive monitoring of ovarian function in Asian elephants (*Elephas maximus*) by measurement of urinary 5 β -pregnanetriol

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The development of an enzymeimmunoassay for 5 β -pregnanetriol and its use for non-invasive monitoring of reproductive cycles in Asian elephants is described. Gas chromatography–mass spectrometry (GCMS) and high performance liquid chromatography (HPLC) confirmed the presence of 5 β -pregnane-3 α ,17 α ,20 α / β -triols as the two most abundant urinary progesterone metabolites. The assay developed used the antiserum anti-5 β -pregnane-17 α ,20 α -diol-3 α -yl glucuronide but was designed to measure the free steroid in urine samples after hydrolysis and extraction. HPLC confirmed the presence of immunoreactive pregnanetriol in urine, but indicated that the measurement was nonspecific. Immunoreactive pregnanetriol concentrations were significantly correlated with the concentrations of both progesterone ($r = 0.98$, $n = 269$, $P < 0.01$) and 17 α -hydroxyprogesterone ($r = 0.95$, $n = 205$, $P < 0.01$), the metabolic precursor of pregnanetriol. The mean $\pm$ SEM deviation of cycles as determined by measurements of plasma progesterone, 17 α -hydroxyprogesterone and urinary pregnanetriol, respectively, were 15.54 ± 1.5 ($n = 23$, where n = number of cycles), 15.21 ± 1.7 ($n = 15$) and 15.45 ± 0.94 weeks ($n = 20$). These results demonstrate that it is possible to monitor ovarian function in Asian elephants by the measurement of urinary immunoreactive pregnanetriol concentrations.

Introduction

The two species of elephant existing today (*Elephas maximus* and *Loxodonta africana*) are classified as endangered (CITES, 1992), with current estimated wild populations of 50 000 and 500 000, respectively. For both species, habitat loss and poaching have been major factors contributing to the decline and fragmentation of the wild populations. At the same time, zoos and private reserves have become increasingly active in the development of management and breeding programmes for animals in captivity, although the establishment of conditions conducive to successful reproduction is often difficult.

During the last 10 years, hormonal methods for monitoring ovarian cycles in both Asian and African elephants have been described. Typically, the duration of an ovarian cycle is 14–16 weeks and is characterized by an 8–10 week luteal phase and a 4–6 week interluteal or follicular phase, according to patterns of circulating concentrations of progesterone (Hess *et al.*, 1983; Brannian *et al.*, 1988; Plotka *et al.*, 1988; Brown *et al.*, 1991; Taya *et al.*, 1991). Concentrations of oestradiol and of LH have also been measured in plasma, but concentrations are generally very low or results inconsistent between studies, and their secretion has not proved useful for indicating ovarian status (Hess *et al.*, 1983; Brannian *et al.*, 1988; Plotka *et al.*, 1988; Mainka and

Lothrop, 1990; Brown *et al.*, 1991; Taya *et al.*, 1991; Gross *et al.*, 1991).

Measurement of circulating concentrations of progesterone therefore provides the most reliable monitor of ovulatory cycles in elephants and as such are widely used in many zoological collections. Unfortunately, the dependence on assessing ovarian function by plasma hormone analysis has been a hindrance for those zoological collections in which collection of blood samples is not possible and cannot be used in studies monitoring the reproductive dynamics of wild animals. There is therefore a need to develop a non-invasive approach for endocrine reproductive assessment in elephants. On the basis of our limited knowledge of elephant ovarian physiology, this should be most feasible by the measurement of urinary progesterone metabolites.

There are no reports of the identity of urinary metabolites of progesterone in elephants. A recent attempt to monitor ovarian function by measuring unmetabolized progesterone in urine from Asiatic elephants was unsuccessful (Mainka and Lothrop, 1990). Although measurement of urinary pregnanediol glucuronide or 20 α -dihydroprogesterone immunoreactivity has provided valuable information on ovarian function in a variety of mammalian species (see, for example, Loskutoff *et al.*, 1986; Kirkpatrick *et al.*, 1990; Hindle *et al.*, 1992; Hodges, 1992 for review), our own unpublished observations (J. Hindle and C. Niemuller) indicated that this did not hold true for Asian elephants. The aims of the present study were therefore to (i) identify the major urinary progesterone metabolite(s) in

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Asian elephants (ii) develop a microtitre plate enzymeimmunoassay for its determination and (iii) evaluate its use as a non-invasive method for monitoring reproduction in this species.

Materials and Methods

Animals and sample collection

Where possible, matched blood and urine samples were collected once a week for 1–3 years from 11 mature female elephants aged 12–25 years from four zoological collections. A 5–10 ml blood sample was collected from either the saphenous vein or an ear vein into heparinized tubes, centrifuged at 2000 *g* for 10 min immediately after collection, and the plasma stored at -20°C . Mid-stream urine samples were collected during the morning, divided into 5 ml aliquots and stored frozen at -20°C .

Oestrous behaviour in the eight females that had access to males was recorded by the elephant keepers on the basis of increased interest by the bull and eventual copulation with the females. Three of the females monitored had no contact with males throughout the duration of the study. Of the eight females that had access to males, three were kept with the bull(s) daily but separated at night. The remaining five cows had continuous access to a bull except during the winter months. During this time, access to the male was restricted to daylight working hours and only for a cow in the latter half of the follicular phase as predicted by analysis of plasma progesterone. As soon as concentrations of plasma progesterone began to rise, the cow had no further contact with the bull.

Creatinine determination

All urine samples were analysed for creatinine concentration after the initial thawing by the method of Hodges and Green (1989) and as previously validated for elephants by Ramsey *et al.* (1981) and Poole *et al.* (1984), to correct for variations in glomerular filtration rate of urine. The assay sensitivity was approximately 0.1 mg ml^{-1} and the intra- and interassay coefficients of variation were 9.3 and 8.8%, respectively ($n = 30$). Urinary creatinine concentration ranged from below detection (samples excluded) to approximately 2 mg ml^{-1} , with a mean of 0.6 mg ml^{-1} .

Gas chromatography mass spectrometry

Ten urine samples (each 20 ml) from three pregnant cows and from seven nonpregnant cows in the mid-luteal ($n = 4$), and follicular ($n = 3$) phase were prepared for gas chromatography mass spectrometry (GCMS) analysis by the method of Shackleton *et al.* (1976). In brief, samples were extracted using a Sep-pack C18 column (Waters, Division of Millipore, UK Ltd, Watford), hydrolysed using sodium acetate hydrolysis buffer (pH = 5) and 25 mg β -glucuronidase-aryl sulfatase (Sigma, Poole, Dorset, sulfatase activity: 12.5 U mg^{-1} solid, β -glucuronidase activity: 1000 U mg^{-1} solid) and then re-extracted via Sep-pack before being dried down on a rotary evaporator. Samples were reconstituted in a 4:1 (v:v) cyclo-hexane:ethanol solution and

separated into three distinct fractions with Sephadex LH-20 (Pharmacia, St Albans, Herts.). The fractions were dried down, reconstituted in ethanol and derivatized with methoxyamine hydrochloride and trimethylsilylimadazole. The final sample was reconstituted in 500 μl cyclohexane of which 1 μl was injected into the gas chromatography mass spectrometer.

Mass spectrometer (MS) profiles thus obtained were scanned and identification of unknown peaks attempted initially by retention times and by computer MS library search (Shackleton *et al.*, 1980). Peaks from gas chromatography (GC) were compared with reference templates and re-run through the GC with straight chain alkanes to determine methylene units for further identification. This was followed wherever possible by coinjection of the unknown peaks with steroid reference standards to determine change in peak height and provide further confirmation of the identity of the steroid metabolite.

Preparation of [^3H]pregnanetriol

As [^3H]pregnanetriols were not commercially available, a [^3H]-labelled form of one of the isomers was prepared in our laboratory by the enzymatic reduction of [^3H]17 α -hydroxy-5 β -pregnane-3 α ,20 α -diol (Amersham, Buckinghamshire) with 3 α -hydroxysteroid dehydrogenase and NADH (method provided by A. P. Scott; Ministry of Agriculture, Fisheries and Food (MAFF), Fisheries Laboratory, Lowestoft). The reagents were incubated for 2 h at room temperature, and the products ether extracted before thin layer chromatography. Preliminary identification of 5 β -pregnane-3 α ,17 α ,20 α -triol as the predominant labelled product was confirmed by recrystallization of the major radioactive peak and pure standard to constant specific activity (SA). The tracer thus prepared had an activity of $8.8\text{ }\mu\text{Ci ml}^{-1}$ and was subsequently used to indicate steroid retention time on HPLC.

HPLC

Unconjugated neutral steroids were separated using HPLC. Sample preparation was done according to the method of Hindle *et al.* (1992). Urine samples (1 ml) were adjusted to pH 5 with hydrolysis buffer and hydrolysed overnight with 1000 FU ($50\text{ }\mu\text{l}$) $^{-1}$ of hydrolysis enzyme (Sigma, sulfatase activity: 4500 U ml^{-1} , β -glucuronidase activity: $100\text{ }000\text{ U ml}^{-1}$). Since conjugated pregnanetriol was not available, efficiency of hydrolysis was monitored by the addition of [^3H]pregnanediol glucuronide ([^3H]PdG) to pooled urine samples monitored separately. Samples were adjusted to pH = 7 with 3 mol NaOH l^{-1} and extracted with 5 ml distilled diethyl ether. Before extraction [^3H]steroids (progesterone, 20 α -dihydroprogesterone, 17 α -hydroxyprogesterone and pregnanetriol) were added to the sample as reference standards and to monitor extraction efficiency. The ether was dried under nitrogen and samples were reconstituted in 0.2 ml of double distilled water:acetonitrile (70:30 v:v).

The progesterone metabolites were separated using a Novapak RP C-18 column ($3.9 \times 150\text{ mm}$, $5\text{ }\mu\text{m}$ particle size; Waters) and an isocratic solvent system of 30:70 acetonitrile:water at a flow rate of 2 ml min^{-1} (B. Junemann, unpublished). Fractions (1 ml) were collected for 36 min, dried and reconstituted in

assay buffer. Mean recoveries for the four tracers were between 71 and 78% ($n = 14$). The overall efficiency of the procedure, including hydrolysis and extraction, was $68.8 \pm 4.2\%$ ($n = 14$) based on the recovery of [^3H]pregnanediol.

Urinary hormone assays

Sample preparation. Urine samples were hydrolysed and extracted before assay. An aliquot of 250 μl of urine was incubated overnight at 37°C with 250 FU ($50 \mu\text{l}$) $^{-1}$ of hydrolysis enzyme (Sigma, sulfatase activity: 4500 U ml^{-1} , β -glucuronidase activity: $100\,000 \text{ U ml}^{-1}$) and 250 μl of hydrolysis buffer. Hydrolysis efficiencies were determined by adding in triplicate tracer amounts of [^3H]oestrone sulfate and oestrone glucuronide to urine pools (250 μl) before hydrolysis. Amounts of unconjugated steroid recovered were $81.1 \pm 6.1\%$ and $84.3 \pm 3.2\%$ (mean $\pm$ SEM, $n = 30$), respectively. The pH was readjusted to 7 with 3 mol NaOH l^{-1} and samples were extracted with diethyl ether and reconstituted in assay buffer. Procedural losses during extraction were monitored by the addition of tracer amounts of [^3H]17 α -hydroxyprogesterone to each sample. Individual recoveries were used to correct for losses when calculating the final hormone concentration. The overall mean $\pm$ SEM extraction was $89.1 \pm 2.1\%$ ($n = 200$).

Pregnanetriol. Immunoreactive 5 β -pregnanetriol was measured using a microtitre plate EIA method similar to that described by Hindle *et al.* (1992). The assay used the antiserum 5 β -pregnane-17,20 α -diol-3 α -yl glucuronide which was raised in rabbit against the immunogenic complex of 5 β -pregnane-17,20 α -diol-3 α -yl glucuronide coupled to BSA (Samarajeewa and Kellie, 1985). The enzyme label, provided by E. Möstl (Institut für Biochemie, Veterinärmedizinische Universität, Vienna), was prepared by conjugating 4-pregnene-17 α ,20 α -diol-3-one-carboxymethylloxime to horse radish peroxidase via the mixed acid anhydride procedure of Lieberman *et al.* (1959). The antiserum, which crossreacted 62.5% with the free steroid as determined by radioimmunoassay (Samarajeewa and Kellie, 1985), was stored in aliquots of 1:1000 in Tris buffer at -20°C while the enzyme label was stored in glycerol (1:1 v:v) also at -20°C .

Microtitre plates (Nunc Immuno 1; Gibco Ltd, Paisley) were coated overnight with sheep anti-rabbit IgG (1 μl per well). The plates were emptied by inversion, filled with Tris buffer (20 nmol l^{-1} ; 0.25 ml per well) containing 0.1% sodium azide and stored at 4°C until needed. Just before use, the plates were emptied, rinsed with a 0.005% Tween solution and blotted dry. Standard (5 β -pregnane 3 α ,17 α ,20 α -triol, Sigma, Poole) was double diluted in Tris buffer over a range of 156 pg ml^{-1} – 10 ng ml^{-1} (7.8 – $500 \text{ pg (50 } \mu\text{l})}^{-1}$) and 0.05 ml aliquots dispensed in duplicate. Samples were diluted in Tris assay buffer and added in duplicate (0.05 ml). Antiserum (1:160 000) and label (1:30 000) were added to all wells except the blanks. Plates were covered and incubated overnight in the dark at 4°C .

After incubation, plates were emptied by inversion, washed with 0.005% Tween solution and tapped dry. Enzyme substrate was prepared immediately before use by adding 0.25 ml hydrogen peroxide solution (30%, 4 mol l^{-1} , BDH) to 100 mg *O*-phenylenediamine; (1,2, benzenediamine; Sigma) in 28 ml of

Fig. 1. Binding inhibition curves demonstrating parallelism in serially diluted Asian elephant urine pool samples from the mid-luteal (Δ) and follicular phase ($\Diamond$) as well as human urine from late pregnancy ($\circ$); pregnanetriol standard ($\bullet$).

a citric acid substrate buffer (0.1 mol citric acid monohydrate l^{-1} ; pH = 5). A volume of 0.25 ml was added to each well; plates were incubated in the dark at 4°C for 45 min and the reaction was stopped by the addition of 0.05 ml of sulfuric acid (3 mol l^{-1}). Absorbance was measured at 492 nm (with a reference filter of dual wavelength at 620 nm) on an automatic plate reader (Dynatech MR 700, Dynatech Laboratories Ltd, Billingham). The amount of immunoreactive pregnanetriol in each well was read from a standard curve constructed by plotting the percentage of sample bound against the amount of standard added.

Pregnanetriol EIA validation. The sensitivity of the assay as determined at 90% binding was 8.5 pg per well (170 pg ml^{-1}). Serial dilutions of elephant urine collected during the mid-luteal and follicular phase and of human late pregnancy urine (a known source of high concentrations of pregnanetriol) gave displacement curves parallel to the 5 β -pregnanetriol standard (Fig. 1). The accuracy of the assay, determined by recovery of unlabelled 5 β -pregnane-3 α ,17 α ,20 α -triol added to elephant urine pools containing low concentrations of endogenous hormone was $98.7\% \pm 1.1\%$ ($n = 10$). The interassay CV for repeated determination of elephant urine pool containing high and low concentrations of pregnanetriol was 10.8 and 5.6% ($n = 30$), respectively, while the intra-assay CV was 7.8% ($n = 30$). Steroids showing crossreactivity determined at 50% inhibition of binding, were 5 α -pregnane-3 β ,17,20 α -triol (109%), 5 β -pregnane-3 α ,6 α ,17 α ,20 α -tetrol (189%), 5 β -pregnane-3 α ,6 α ,17 α ,20 β -tetrol (113%), 5 α -pregnane 3 β ,17 α ,20 β -triol (106%), 5 β -pregnane-3 α ,17 β ,20 β -triol (2.9%), progesterone (1.45%), 5 β -pregnanediol (1.1%). All of the other C21, C19 and C18 steroids tested crossreacted less than 1%. Specificity of measurement of pregnanetriol in Asian elephant urine was determined by co-chromatography on HPLC. The presence of pregnanetriol immunoreactivity co-eluting with the 5 β -pregnanetriol tracer (Fig. 2) confirmed that the assay could detect pregnanetriol in urine from Asian elephants. However, the presence of large amounts of immunoreactivity in fractions preceding the peak of [^3H]pregnanetriol indicated that the measurement of pregnanetriol was not specific. The values

Fig. 2. Co-chromatography profiles from HPLC of immunoreactive progesterone metabolites in Asian elephant urine. The elution profile (●) of pregnanetriol immunoreactivity in hydrolysed and extracted urine is compared with that of [^3H]-labelled pregnanetriol (P3), 20 α -dihydroprogesterone (20A), pregnanediol (PDIOL) and progesterone (P4) (○).

reported here are therefore expressed as pregnanetriol immunoreactivity.

20 α -Dihydroprogesterone ELA. The assay was as described by Hindle *et al.* (1992). Immunoreactive 20 α -dihydroprogesterone was measured in fractions collected from the HPLC. The assay using an anti-4-pregnene-20 α -ol-3-one-carboxymethyloxime antiserum and enzyme conjugate of horse radish peroxidase conjugated to 20 α -dihydroprogesterone (provided by E. Möstl) showed crossreactivities with 5 β -pregnane-20 α -ol-3-one (11.9%), 5 β -pregnane 3 α -ol-20-one (0.92%), 5 β -pregnane-3 α -20 α diol (0.57%), progesterone (0.52%), pregnenolone (<0.1%) and 5 β -pregnane-3 α ,17 α ,20 α -triol (<0.01%). The assay sensitivity as determined at 90% binding was 150 pg ml $^{-1}$. The intra-assay CV was 8.4% ($n = 30$), whereas the interassay CV was 12.1 and 16.1% ($n = 15$) for high and low value quality control pools, respectively.

Assays of plasma hormone

Progesterone. Progesterone was measured by radioimmunoassay as initially described by Shaw *et al.* (1989) with modifications. The assay used 1,2,6,7- ^3H progesterone (Amersham) and sheep anti-progesterone 11 α -hemisuccinate-ovalbumin (from J. Foulkes, MAFF, Shinfield, Reading) as antiserum which showed the following crossreactivities: 11 α -hydroxyprogesterone (29.8%), 11 β -hydroxyprogesterone (16.5%), 5 β -pregnanedione (16.1%), 5 α -pregnanedione (2.63%) and less than 0.1% with all other steroids tested. Samples (500 μl) were extracted with petroleum ether (recovery $83.6 \pm 2.3\%$, $n = 200$) and reconstituted in assay buffer. The sensitivity of the assay at 90% binding was 20 pg ml $^{-1}$; intra- and interassay CVs were 8.9% and 10.7%, respectively. Serial dilutions of extracted elephant plasma gave displacement curves parallel to the progesterone standard. The accuracy of the assay determined by the mean $\pm$ SEM recovery of unlabelled progesterone added to extracted plasma pools of low endogenous hormone was $99.4 \pm 2.5\%$.

17 α -Hydroxyprogesterone. The measurement of 17 α -hydroxyprogesterone in plasma and HPLC samples followed the

same protocol as used for the progesterone radioimmunoassay. The assay used 1,2,6,7- ^3H 17 α -hydroxyprogesterone (Amersham) with an anti-sheep-17-hydroxyprogesterone-3-carboxymethyloxime antiserum (Steranti, St Albans). The antiserum crossreacted with progesterone (2.2%) and 5 α -pregnanedione (1.1%) as determined by Steranti. Other C21 and C19 steroids tested crossreacted less than 1%. Samples (500 μl) were extracted with petroleum ether (recovery $87.6 \pm 3.1\%$, $n = 200$) and reconstituted in assay buffer. The sensitivity of the assay based on 90% binding was 20 pg ml $^{-1}$, while intra- and interassay CVs were 6.3 and 9.7%, respectively. Serial dilutions of samples extracted from plasma of pregnant and mid-luteal phase elephants gave displacement curves parallel to the 17 α -hydroxyprogesterone standard. The accuracy of the assay determined by the mean $\pm$ SEM recovery of unlabelled 17 α -hydroxyprogesterone (10–160 pg ml $^{-1}$) added to extracted plasma pools of low endogenous hormone was $101.7 \pm 1.4\%$.

Analysis of data

Stages of the ovarian cycle. The definition for the luteal and follicular stages of the elephant reproductive cycles based on analysis of plasma hormones for this study was modelled after the definition originally described by Plotka *et al.* (1988). For both progesterone and 17 α -hydroxyprogesterone, the onset of the luteal phase of the cycle was defined as the first point (after the respective plasma concentrations fell below 200 pg ml $^{-1}$) that increased by 50 pg and remained high for at least two weeks; the end of the luteal phase was defined as the first of two consecutive values within 50 pg that were less than 200 pg and at least 50 pg lower than the preceding value. The composite data presented were therefore aligned to the onset of the luteal phase (week 1) according to this definition.

Similarly, according to urinary pregnanetriol concentrations, the onset of the luteal phase was defined as the first point after a fall in values below 200 ng mg $^{-1}$ creatinine which increased by 50 ng mg $^{-1}$ creatinine and remained high for at least two weeks. Similarly, the end of the luteal phase was taken as the first of two consecutive values within 50 ng mg $^{-1}$ creatinine, which were less than 200 ng mg $^{-1}$ creatinine and at least 50 ng mg $^{-1}$ creatinine lower than the preceding value.

The duration of the ovarian cycle was determined as the interval between the onset of two successive luteal phases as defined separately for each hormone measured.

Statistical analysis

Statistical analysis was carried out by standard analyses of mean and SEM and Student's *t* test as appropriate.

Results

According to the mass spectra obtained, the silyl ether derivatives of 5 β -pregnanetriol (as described by Quilliam and Westmore, 1980) were the most abundant of the progesterone metabolites successfully identified. 5 β -Pregnane-3 α ,17 α 20 α / β -triols were present in every urine sample analysed from

Table 1. Summary of results of gas chromatography-mass spectrometry of urine from Asian elephants

Reproductive state	Identified steroid metabolite	Incidence
Follicular (<i>n</i> = 3)	11 β -hydroxyandrosterone/aeticholanolone	1
	No detectable pregnanetriols	3
Mid-luteal (<i>n</i> = 4)	5 β -pregnane-3 α ,17 α ,20 α -triol	4
	5 β -pregnane-3 α ,17 α ,20 β -triol	4
	5 β -pregnane-3 α ,6 α ,20 α -triol	2
	5 α -pregnane-3 α ,11 β ,20 α -triol	1
	5 α -pregnane-3 β ,17,20 α -triol	1
	3 α ,20 α dihydroxy-5 β -pregnan-11-one	1
	11 β -hydroxyandrosterone/aeticholanolone	1
Pregnancy (<i>n</i> = 3)	5 β -pregnane-3 α ,17 α ,20 α -triol	3
	5 β -pregnane-3 α ,17 α ,20 β -triol	3
	5 α -pregnane-3 β ,17,20 α -triol	1
	5 α -pregnane-3 α ,11 β ,20 α -triol	1
	3 α ,20 α dihydroxy-5 β -pregnan-11-one	2

pregnant Asian elephants and those at the mid-luteal phase (Table 1). Quantitatively, these two steroid metabolites were also the most abundant forms of pregnanetriol present (data not shown). It was not possible to detect any pregnanetriol in urine from the follicular phase, and progesterone, pregnanediol and 20 α -dihydroprogesterone were not detected in any samples.

The results of HPLC analysis of a sample of hydrolysed and extracted urine from Asian elephants at the mid-luteal phase are shown (Fig. 2). Immunoreactivity co-eluting with the [3 H]pregnanetriol marker was found in all samples tested (*n* = 6), thus indicating the presence of immunoassayable pregnanetriol in urine of Asian elephants at the luteal phase. As indicated earlier, relatively large amounts of additional immunoreactivity not co-eluting with the [3 H]pregnanetriol label was also present in fractions 4–9 in all samples analysed (*n* = 6). In contrast, progesterone, 20 α -dihydroprogesterone and 17 α -hydroxyprogesterone were not detectable in HPLC fractions by their specific assays at the volumes and dilutions used.

The profile of urinary pregnanetriol immunoreactivity in relation to that of circulating progesterone concentrations during three consecutive ovarian cycles in one elephant is shown (Fig. 3). The urinary measurements revealed a cyclic pattern of excretion in which the luteal and interluteal periods, as determined by intervals of high and low circulating progesterone concentrations, could be clearly distinguished. In this animal, maximum pregnanetriol concentrations at the luteal phase were between 300 and 750 ng mg $^{-1}$ creatinine, approximately five-fold greater than those of the corresponding interluteal period. There is a close temporal relationship between the defined rise in plasma progesterone and urinary pregnanetriol following predicted ovulation in each of the three cycles illustrated.

Composite profiles of the mean and SEM of plasma concentrations of progesterone (*n* = 11 animals, *n* = 23 cycles) and urinary concentrations of pregnanetriol (*n* = 8 animals, *n* = 20 cycles) throughout the ovarian cycle in Asian elephants are shown (Fig. 4). Corresponding data for plasma 17 α -hydroxyprogesterone, the major circulating precursor for preg-

Fig. 3. Representative reproductive profile of weekly (a) plasma progesterone and (b) urinary pregnanetriol concentrations for one female Asian elephant through four consecutive reproductive cycles and into early pregnancy. (O: predicted ovulation; M: mating; C: conception).

nanetriol (Axelrod and Goldzieher, 1960), are also shown from 15 of these cycles (*n* = 8 animals). All data were aligned to the rise in plasma progesterone reflecting the start of the luteal phase. The overall duration of the oestrous cycle (mean $\pm$ SEM) calculated from the interval between successive luteal phases was 15.54 ± 1.5 (*n* = 23), 15.21 ± 1.7 (*n* = 15) and 15.45 ± 0.94 (*n* = 20) weeks, based on measurements of plasma progesterone, 17 α -hydroxyprogesterone and urinary pregnanetriol, respectively. Immunoreactive pregnanetriol concentrations were significantly correlated with the concentrations of both progesterone ($r = 0.98$, *n* = 269 cycles, $P < 0.01$)

Fig. 4. Mean ($\pm$ SEM) profiles of weekly concentrations of (a) progesterone, (b) 17 α -hydroxyprogesterone and (c) pregnanetriol samples for Asian elephants ($n = 11$) representing one reproductive cycle. All data were aligned to the elevation in plasma or urinary steroid concentrations (week 1) until the following week 1.

and 17 α -hydroxyprogesterone ($r = 0.95$, $n = 205$ cycles, $P < 0.01$) throughout the ovarian cycle. Plasma concentrations of 17 α -hydroxyprogesterone were also significantly correlated with circulating concentrations of progesterone during the ovarian cycle ($r = 0.93$, $n = 205$ cycles, $P < 0.01$).

The data show clear cyclic patterns of pregnanetriol excretion, with values increasing from an overall mean of 45 ng mg⁻¹ creatinine (range 5–172 ng mg⁻¹ creatinine) during the interluteal period to a mean of 237 ng mg⁻¹ creatinine in the luteal phase (range 75–802 ng mg⁻¹ creatinine). Although variability was seen in pregnanetriol values, both between cycles and between animals, the extent of this was not markedly different from that seen in measurements of plasma hormones (as evidenced by the size of the SE bars in Fig. 3). Most importantly the trend of high urinary pregnanetriol concentrations throughout the interluteal period was maintained in all profiles and of the 20 cycles with matching progesterone and pregnanetriol data, defined rises in both hormones occurred on the same date 18 times (90%). In the remaining two cycles, the rises were separated by one week (for example, one sample apart).

Table 2. Relationship between predicted oestrus and mating events in six Asian elephants from one zoo from January 1989 – November 1991

Total number of cycles	Mating matching predicted oestrus (%)	No mating with predicted oestrus		Resulting pregnancy
		Wrong time (%)	No mating (%)	
8	4 (50)	1 (12.5)	3 (37.5)	1
11	4 (36.4)	4 (36.4)	3 (27.3)	0
6	3 (50)	1 (16.7)	2 (33.3)	0
8	3 (37.5)	1 (12.5)	4 (50)	0
8	2 (25)	3 (37.5)	3 (37.5)	2
4*	1 (25)	0 (0)	3 (75)	0
45	17 (37.8)	10 (22.2)	18 (40)	3
Overall conception rate: 3/17 $\times$ 100 = 17.6%				

*This cow completed a full term pregnancy in July of 1990.

In those cycles in which mating did occur (Fig. 3), only during the last (conception) cycle did mating correspond to the time of predicted ovulation resulting in pregnancy. In all of the other cases, mating either did not occur at all, or it occurred at least a week before the time of expected ovulation and did not result in conception. Furthermore, the apparent asynchrony between behavioural and endocrine (physiological) events was not confined to this individual, but was seen in all six adult females in the group to which she belonged (Table 2). The data were collected for 34 months and indicated that, out of a total of 45 ovarian (presumed ovulatory) cycles from six adult cows, mating coincided with the time of predicted ovulation in only 17 (37.8%) cases. In the remaining 62.2% of the cycles, mating either did not occur (40%) or occurred at a time separated from presumed ovulation (22.2%). Of the 17 cycles in which mating appeared to coincide with ovulation, three resulted in conception (17.6%). No pregnancy occurred from the mis-timed matings.

Discussion

This study has shown that 5 β -pregnanetriol is a major urinary progesterone metabolite in Asian elephants. The establishment of an enzymeimmunoassay for the measurement of immunoreactive pregnanetriol has enabled the first description of the pattern of excretion of urinary progesterone metabolites throughout the ovarian cycle. The results suggest that measurement of pregnanetriol should provide a useful non-invasive method for monitoring reproductive status in this species.

The initial GCMS findings of relatively high concentrations of 5 β -pregnanetriol in urine of female Asian elephants during various reproductive states was unusual, but may explain the previous lack of success in attempting to monitor reproductive cycles with more commonly measured progesterone metabolites such as 20 α -dihydroprogesterone and pregnanediol glucuronide. Measurement of urinary pregnanediol or 20 α -dihydroprogesterone have proved to be invaluable for monitoring reproductive

status in a wide variety of species including black and white rhinos (Hindle *et al.*, 1992), gorillas (Hodges and Green, 1989), giant pandas (Hodges *et al.*, 1984), white-tailed deer (Knox *et al.*, 1992), killer whales (Walker *et al.*, 1988) and macaques (Monfort *et al.*, 1986). In contrast, however, the immunoreactivity associated with these metabolites in Asian elephant urine is low and variable and their patterns of excretion do not correlate with ovarian events (J. Hindle and C. Niemuller, unpublished). Furthermore, in all samples analysed, neither progesterone nor its metabolites, pregnanediol and 20 α -dihydroprogesterone, were detected by the GCMS procedures used. In an independent study, Mainka and Lothrop (1990) attempted but failed to measure urinary progesterone cycles in two Asian elephants, a finding consistent with the above results.

Pregnanetriol is a unique major urinary metabolite of 17 α -hydroxyprogesterone produced by reductions in ring A, C-3 and C-20 (Gower and Honour, 1984). Thus, the formation of pregnanetriol follows a separate pathway from that used in the production of 20 α -dihydroprogesterone and pregnanediol (Gower and Honour, 1984), for which 17 α -hydroxyprogesterone is not an obligatory intermediate (Axelrod and Goldzieher, 1960).

The measurement of pregnanetriol, originally discovered in the urine of two women with congenital adrenal hyperplasia (Butler and Marrian, 1937), is normally used as an indicator of adrenal hyperplasia rather than for monitoring ovarian function. Human ovaries, however, are known to be a source of its precursor, 17 α -hydroxyprogesterone (Zander, 1958, Short and London, 1961) and measurement of pregnanetriol for determining ovarian function in women has been reported (for example Pickett *et al.*, 1959; Fotherby, 1960; Pickett and Kellie, 1962).

The results reported here are of nonspecific measurements of unconjugated pregnanetriol immunoreactivity, as HPLC data clearly indicated the presence of other, more polar immunoreactive substances. These unidentified substances may even be quantitatively more important than pregnanetriol; however, their nature remains unknown. Although the antibody cross-reacts extensively with other pregnanetriol isomers and with pregnanetetrols, all of which can be expected to elute from HPLC at positions similar to that of the unknown, their contribution to the immunoreactive profiles described cannot be determined.

The nature of the relationship between urinary pregnanetriol and plasma 17 α -hydroxyprogesterone prompted the initial measurements of 17 α -hydroxyprogesterone throughout the ovarian cycle of Asian elephants in this study. The significant correlation of circulating plasma concentration of 17 α -hydroxyprogesterone with progesterone suggests that the elephant ovary is a major source of this steroid and that urinary pregnanetriol concentrations are related to ovarian progesterone secretion. In other mammals, luteal phase 17 α -hydroxyprogesterone concentrations are much lower than progesterone concentrations. Furthermore, the significant correlation between pregnanetriol and both progesterone and 17 α -hydroxyprogesterone throughout the reproductive cycle provides additional evidence that pregnanetriol is a major urinary progesterone metabolite in elephants and that its pattern of excretion reflects ovarian function. The results presented here suggest that elephants may have significantly higher 17 α -hydroxylase

activity in both the ovary and the liver, which may explain why pregnanetriol is the major urinary progestagen metabolite.

The general features of the reproductive cycles described in this study were based upon reference measurements of circulating concentrations of plasma progesterone. Thus, the cyclic changes in the pattern of plasma progesterone concentrations indicated that the duration of the reproductive cycle of the 11 Asian elephants in this study ranged between 14 and 18 weeks, which matched with earlier descriptions (Hess *et al.*, 1983; Plotka *et al.*, 1988; Brown *et al.*, 1991; Taya *et al.*, 1991). Data presented from both the individual as well as the composite profiles revealed a cyclic pattern of pregnanetriol excretion highly correlated with circulating plasma progesterone and 17 α -hydroxyprogesterone concentrations. Despite higher variability in urinary values as compared with plasma steroid concentrations, there was a clear fivefold increase in luteal phase values compared with interluteal phase values. Furthermore, 90% of the cycles analysed demonstrated an increase in pregnanetriol concentrations concurrent with the defined rise in plasma progesterone. Although plasma progesterone has to date been the most useful measurement in determining reproductive cycles, these results demonstrate that the measurement of pregnanetriol closely reflects progesterone secretion and corpus luteum function.

Although it is generally assumed that cyclic fluctuations in the concentrations of circulating progesterone (or its metabolites) reflect ovulatory cycles, there is no information on the elephant from which a cycle can be defined as endocrinologically or behaviourally normal. The results presented here indicate that in individuals from one particular group, only 25–50% of apparently 'normal' ovulatory cycles were associated with mating behaviour during the periovulatory period, despite access to bulls of proven fertility throughout this time. Furthermore, of the cycles in which mating did occur at the time of expected ovulation, less than 20% resulted in conception. In domestic species such as cows, horses, goats and sheep, conception rate per ovulatory event is higher, varying between 40 and 75% (Bristol, 1986; McDonald, 1986; Smith, M. C., 1986; Smith, R. D., 1986). Data collected by researchers on wild African elephants in Amboseli National Park, Kenya, have noted a conception rate of approximately 75% (Moss, 1983). At this time, the reason for the high percentage of non-fertile cycles in the captive elephant population is unclear. Our data demonstrate that in only a small proportion of cycles does mating occur at the time of presumed ovulation. This behavioural/physiological imbalance may be related to the highly variable pattern of oestrogen secretion during the cycle and the inability in most studies to describe a clear preovulatory increase in oestrogens (Hess *et al.*, 1983; Mainka and Lothrop, 1990; Brown *et al.*, 1991; Taya *et al.*, 1991). Furthermore, with the exception of the studies of Hess *et al.* (1983) and Mainka and Lothrop (1990), the above studies lacked the matching behavioural data for their endocrinological descriptions of the elephant reproductive cycle. These two studies indicated that the prevalence of non-fertile cycles in the present study group of cows was not an isolated event. Mainka and Lothrop (1990) described flehmen but not mating events of a bull towards a cow (who did not conceive) through five complete cycles and comment on three mating times of February, July and October in a second cow before conception occurred. Likewise, Hess

et al. (1983) described 15 reproductive cycles divided between six cows, from which only two conceptions occurred. The reason for the apparent infertility is not understood.

The incidence of asynchronous mating with respect to presumed ovulation observed in this study does not appear to have been described before. The lack of mating or mistiming of mating may have been overestimated as animals were not constantly observed over 24 h, thus matings occurring at night would not have been observed. The most important external cue received by the male from the female signifying onset of oestrus appears to be smell as determined by the flehmen responses (Jainudeen *et al.*, 1971; Hess *et al.*, 1983). Miscoeing of these pheromonal signals on the part of the female may be another reason for mis-timed mating events. There may be a link between the timing of ovulation and miscoeing of pheromonal signals, which might explain the asynchronous mating events.

In summary, this paper has identified 5 β -pregnanetriol as a major urinary progesterone metabolite in Asian elephants and described its measurement throughout the ovarian cycle of this species. Its measurement in urine should not only enable routine, non-invasive monitoring of reproductive cycles in captivity, but also provide potential application towards assessing reproductive status in wild Asian elephants.

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