Demonstration of 2-unsaturated C₁₉-steroids in the urine of female Asian elephants, *Elephas maximus*, and their dependence on ovarian activity

M. Dehnhard¹, M. Heistermann², F. Göritz¹, R. Hermes¹,
T. Hildebrandt¹ and H. Haber³

¹Institute for Zoo Biology and Wildlife Research, PF 601103, D-10252 Berlin, Germany;

²Department of Reproductive Biology, German Primate Centre, Kellnerweg 4, D-37077

Göttingen, Germany; and ³Institute of Pharmacy, Humboldt University, Goethestr. 54, D-13086 Berlin, Germany

Air-borne volatile substances have been demonstrated to signal oestrus, induce ovulation and synchronize ovarian activity in different mammals. An oestrous-related pheromone of the female Asian elephant (*Elephas maximus*) is known to induce behavioural responses in elephant bulls. Additional data revealed that timing of oestrus in females with close social relationships tends to be synchronized. Therefore, urine from female Asian elephants might be expected to contain luteal phase-dependent volatile substances, which may function as additional chemical signals in this species. The aim of the present study was to identify such compounds and to investigate their pattern of excretion throughout the ovarian cycle. Urine samples were collected three times a week during the follicular phase and one to three times a week during the luteal phase from five adult female Asian elephants from a total of 13 non-conception cycles and one conception cycle, including the first 72 weeks of pregnancy. A simple headspace solid-phase microextraction method has been developed for quantification of urinary volatile substances and analysis was performed by

gas chromatography. The comparison of urine collected during the follicular and the luteal phase indicated the presence of two luteal phase-dependent substances. Mass spectrometry was used to identify one substance as 5 α -androst-2-en-17-one and a second substance as the corresponding alcoholic compound 5 α -androst-2-en-17 β -ol. The 5 α -androst-2-en-17 β -ol and -17-one profiles reflected cyclic ovarian activity with clear (10–20-fold) luteal phase increases. Furthermore, measurements of both compounds were correlated positively with the concentration of urinary pregnanetriol and indicated cycle duration (15.1 ± 1.2 weeks) similar to that obtained from pregnanetriol measurements (15.2 ± 1.6 weeks). The results demonstrate the presence of two luteal phase-specific steroidal volatile compounds in elephant urine. One of the substances, 5 α -androst-2-en-17-one, has been demonstrated in human axillary bacterial isolates. The measurement of both volatile substances in elephant urine can be used for rapid detection of the stage of the ovarian cycle, as the analysis can be completed within 2 h.

Introduction

Traditionally, immunoreactive progesterone or 5 α -reduced gestagen measurements in blood have been used to monitor ovarian cycles in Asian and African elephants (Hess *et al.*, 1983; Plotka *et al.*, 1988; Heistermann *et al.*, 1997; Hodges *et al.*, 1997; Dehnhard *et al.*, 1998). On the basis of profiles generated by these studies, a cycle duration of 13–17 weeks has been established for both species, consisting of a 4–6 week follicular phase and a 9–11 week luteal phase (for review, see Hodges, 1998). More recently, non-invasive techniques based on the analysis of urinary and faecal progestin metabolites have also been developed (Niemüller

et al., 1993; Wasser *et al.*, 1996; Heistermann *et al.*, 1997; Fieß *et al.*, 1999).

An alternative approach to gaining information on female reproductive status and, in particular, determining the stage of the oestrous cycle in Asian elephants might be the measurement of pheromones. Pheromones are olfactory stimuli that release specific behavioural or endocrine reactions in the recipient (Karlson and Lüscher, 1959). Behavioural effects of oestrous-related pheromones, such as attraction, olfactory preferences or flehmen responses of males to urine and vaginal secretions from females during oestrus have been described in several species, including rats (Carr *et al.*, 1966), mice (Schwende *et al.*, 1984; Kavaliers *et al.*, 1994; Kavalier and Kinsella, 1995), hamsters (Singer *et al.*, 1976), sheep (Blisset *et al.*, 1990, 1994), cows (Dehnhard *et al.*, 1991), cotton-top tamarins

Email: dehnhard@izw-berlin.de

(Ziegler *et al.*, 1993) and Asian elephants (Rasmussen *et al.*, 1997), but the chemical nature of the substances involved is mostly unknown. The Asian elephant is one of the few vertebrate species in which the identity of a compound that functions as an oestrous-related pheromone has been determined. The sex pheromone (Z)-7-dodecenyl acetate, which is secreted with the urine during the follicular phase and reaches maximum concentrations just before ovulation, elicits a chemosensory response, the flehmen reaction, in elephant bulls (Rasmussen *et al.*, 1997).

In addition to oestrous-related pheromones, there is evidence for gestagen-dependent pheromones in several vertebrate taxa, including fish, reptiles and mammals. In fish, active pheromonal oestrogen and gestagen metabolites are released into the water (Scott and Sorensen, 1994; Sorensen *et al.*, 1998). Exposure to the pheromone 17 α ,20 β -dihydroxy-4-pregnen-3-one enhances behavioural spawning success, sperm production and sperm motility of male goldfish (Defraipont and Sorensen, 1993). A family of diene-related (3,7-diethyl-9-phenyl-2-nonanone)-related steroidal esters has been isolated from crocodile paracloacal glands and shown to have probable nest site attraction properties (Whyte *et al.*, 1999; Yang *et al.*, 1999). In rabbits, progesterone administration effectively reinstates the emission of the nipple pheromone in ovariectomized animals (Gonzales-Mariscal *et al.*, 1994). These examples support the idea that there are gestagen-dependent substances serving as chemical signals in elephants.

Therefore, the objective of this study was to investigate the presence of luteal phase-related volatile substances in the urine of Asian elephants, which might be available as chemosignals for other elephants. In view of their possible function in communicating reproductive status (for example, for synchronization of ovarian cycles or for purposes of mate choice), chemosignals may represent more informative indicators of behavioural and physiological condition than measurements of circulating or excreted hormones. Furthermore, the measurement of luteal-related volatile substances may also be useful for practical purposes of timing reproductive events during the oestrous cycle. In both respects, identification of such compounds is a prerequisite for further studies on their possible chemosensory role and usefulness in monitoring female reproductive function. The specific aims of the present study were therefore: (i) to use the solid-phase microextraction technique (SPME; Arthur and Pawliszyn, 1990) in combination with gas chromatography-mass spectrometry (GC-MS) analysis to identify luteal-phase related volatile compounds in the urine of Asian elephants; and (ii) to determine their pattern of excretion throughout the ovarian cycle in relation to urinary progesterone metabolites.

Materials and Methods

Animals and sample collection

Five adult female Asian elephants aged 12–29 years were involved in the study and were maintained in mixed social

groups in the zoos of Leipzig and Berlin. All animals were kept outside during the day and housed in separate boxes at night. None of the females had reproduced successfully before the study. Urine samples were collected from 1998 to 1999, three times a week during the follicular phase and one to three times a week during the luteal phase. Samples were obtained from a total of 13 non-conception cycles and one conception cycle plus the first 72 weeks of pregnancy. In one of the animals, daily urine samples were collected from two follicular phases. The samples were collected in the morning by placing a 20 ml glass tube in the mid-stream of the urinating animal for the analysis of volatile substances and in plastic tubes for enzymeimmunological measurements of urinary 5 β -pregnanetriol. All samples were frozen immediately after collection and stored at –20°C until analysed. Freezing at –20°C for 4 months and incubation (4 h at 37°C) did not result in a significant loss of 2-unsaturated C₁₉-steroids.

Determination of female reproductive status

Immunoreactive 5 β -pregnanetriol, an abundant urinary progesterone metabolite in Asian elephants, was measured using the method of Niemüller *et al.* (1993) to determine female reproductive status and the stage of cycle. Samples were diluted 1:200 in assay buffer (0.04 mol PBS l⁻¹, containing 0.1% BSA, pH 7.0) and 50 μ l aliquots were analysed directly using a microtitreplate enzymeimmunoassay. The assay used an antiserum raised in a rabbit against 5 β -pregnane-17,20 α -diol-3 α -yl-glucuronide-BSA and biotinylated 5 β -pregnanetriol as a label. Crossreactivity values of the antibody with different steroids are given by Niemüller *et al.* (1993). Sensitivity of the assay at 90% binding was 15 pg. Intra- and interassay coefficients of variation of high and low value urine quality control pools were 6.3 and 9.4% (high) and 8.9 and 11.7% (low), respectively.

Urine samples were analysed for concentrations of creatinine (Cr) using the method described by Heistermann and Hodges (1995) to control for differences in urine concentration. Urinary hormone concentrations are expressed as mass per mg creatinine.

Solid-phase microextraction technique sampling

SPME sampling was carried out using a fibre with an 85 μ m polyacrylate coating, held in a syringe-like manual sampling device (Supelco, Bellefonte, PA). An aliquot of 4.5 ml urine, 0.5 ml 2 mol Tris buffer l⁻¹ (pH 7.0), 1.83 g NaCl, and 500 ng 5 α -androstane (Sigma, Deisenhofen) as internal standard were added into 20 ml headspace vials (Perkin Elmer, Norwalk, CT). The vials were sealed with a headspace aluminium cap with a PTFE-coated silicone rubber septum (Perkin Elmer). The septum of the vial was pierced in the centre to facilitate the insertion of the SPME needle. The vial was then heated thermostatically to 100°C and agitated with a magnetic stirring bar. The needle was pushed through the septum and the fibre was exposed by

depression of the plunger. The end of the fibre was located approximately 20 mm above the surface of the urine. Preliminary tests revealed that the recovery of both steroids increased as the heating temperature increased from 40° to 100°C. The substances were poorly detectable below 60°C, and maximum amounts were obtained at 100°C. After an adsorption time of 60 min, the fibre was retracted into the needle and the holder was withdrawn.

The needle was inserted into the injector of the gas chromatograph; the fibre was extended by depression of the plunger and chromatographic analysis was started. The analytes were desorbed at 300°C; desorption was carried out in the splitless mode, and the fibre was maintained for approximately 15 min in the chromatograph injector.

Analyses were carried out in a Perkin Elmer (PE) Auto-System XL gas chromatograph provided with a flame ionization detector (FID), a PE Nelson 900 Series Interface and a TC4 Turbochrom Chromatography workstation. A 30 m × 0.25 mm inner diameter (0.25 µm film thickness) PE-5 coating fused-silica capillary column (PE) was used. The GC oven was kept at 50°C for 5 min, increased to 240°C at 5°C min⁻¹ and then from 240 to 310°C at 10°C min⁻¹. The carrier gas was nitrogen at 140 kPa. The detector was kept at 280°C.

Gas chromatography–mass spectrometry (GC–MS)

GC–MS determinations were conducted with a Fisons Trio 1000 GC–MS data system. The samples were analysed using a 30 m DB–5MS capillary column (0.32 i.d. and 0.12 µm film thickness; J & W Scientific, Folsom, CA). Ultrapure helium was used as carrier gas, with a head pressure setting of 150 kPa. Injector temperature was 300°C; the interface and ion source temperature were maintained at 300 and 200°C, respectively. Splitless injection mode was used; the purge valve was turned on 3 min after injection, with a split flow of 25 ml min⁻¹ during the GC run. The GC oven was kept at 50°C for 5 min, and increased to 300°C at 10°C min⁻¹. Electron-impact ionization mass spectra were recorded in the full scan mode. The mass spectra were identified by computer MS library research and compared with those of the authentic standards.

Quantification

At the beginning of the study, the FID peak area of the major compound 5α-androst-2-en-17β-ol was integrated quantitatively using the chromatography workstation. When 5α-androst-2-en-17β-ol was identified as the dominant 2-unsaturated steroid, a calibration line was prepared by adding various amounts of 5α-androst-2-en-17β-ol (Steraloids, Newport, RI; 10, 25, 50, 75, 100, 250, 500, 750 ng ml⁻¹) and a constant amount of 100 ng 5α-androstan ml⁻¹ to pooled urine samples (three replicates) from the follicular phase. These urine samples had previously been shown to contain no detectable amounts of 5α-androst-2-en-17β-ol, 5α-androst-2-en-17on and 5α-

androstan. A calibration curve for the relative peak area of 5α-androst-2-en-17β-ol to the internal standard (5α-androstan) versus the concentration of 5α-androst-2-en-17β-ol was obtained. The calibration curve showed a good linearity in the range of 10–750 ng ml⁻¹. The equation for the straight line was $y = 0.236 - 0.015x$ ($r^2 = 0.999$). Urinary concentrations were calculated from the peak ratios according to the above equation. The concentrations of the minor compound 5α-androst-2-en-17-one was calculated by relating the peak area of the samples to 100 ng 5α-androst-2-en-17-one ml⁻¹ added to urine (five replicates).

Reproducibility, recovery and sensitivity of the 5α-androst-2-en-17β-ol determination

The reproducibility of headspace SPME analyses was calculated from repeated measurements of two samples. The coefficients of variation were 5.3% (110.7 ± 5.9 ng ml⁻¹; $n = 6$) and 23.9% (27.3 ± 6.5 ng ml⁻¹; $n = 6$), respectively. For the recovery, 100 ng 5α-androst-2-en-17β-ol ml⁻¹ was added to pooled urine previously shown to contain no detectable amounts of the analyte. The recovery was 114% (113.8 ± 16.9 ng ml⁻¹; $n = 5$). The detection limit for the determination of 5α-androst-2-en-17β-ol, at a signal-to-noise ratio of 2:1, was found to be 5 ng ml⁻¹.

Statistical analysis

The date of ovulation was determined by an increase in urinary pregnanetriol concentrations above a given threshold value (mean + 2 SD) of the preceding follicular phase values (Heistermann *et al.*, 1997). The same criteria were applied to 5α-androst-2-en-17β-ol concentrations evaluated as FID response or concentration. Spearman's rank correlation coefficient was used to determine the association between concentrations of urinary pregnanetriol and 5α-androst-2-en-17β-ol in each individual and 5α-androst-2-en-17-one for one individual.

Results

Comparison of profiles of volatile substances between oestrous and dioestrous urine

Comparison of profiles of volatile substances between urine collected at oestrus and dioestrus revealed several unknown putative follicular phase-specific compounds with high inter- and intra-individual variance of their quantities. However, all animals showed two luteal phase-specific high boiling-point compounds, eluting from the column and separated by 0.2 min when a temperature of approximately 250°C was reached.

Identification of two luteal phase-dependent compounds

According to the mass spectra obtained, the major compound was identified as 5α-androst-2-en-17β-ol (Fig. 1a). The minor compound eluting 0.2 min earlier was identified as 5α-androst-2-en-17-one (Fig. 1c). The mass

Fig. 2. Profiles of (a) pregnanetriol, the most abundant urinary progesterone metabolite in the Asian elephant (*Elephas maximus*), and (b) 5 α -androst-2-en-17 β -ol in urine during five complete ovarian cycles in an individual female elephant. Pregnanetriol was measured with an enzyme immunoassay. 5 α -androst-2-en-17 β -ol was obtained using the headspace solid-phase microextraction (SPME) technique, with the flame ionization detector (FID) response (area $\times 10^6$) proportional to the concentrations.

spectra of both substances obtained were similar to those of their synthetic compounds (Fig. 1b,d). 5 α -androst-2-en-17 β -ol and 5 α -androst-2-en-17-one showed base peak values at m/z 220 and m/z 218, respectively, resulting from the presence of the double bond in the C-2 position. This double bond directs a retro Diels–Alder fragmentation of the A ring with charge retention on the remaining steroid moiety (Labows *et al.*, 1979). The relative retention times of the suspected peak values (1.117 and 1.122 in relation to the internal standard 5 α -androstan) were found to be identical to those of the standards of 5 α -androst-2-en-17on and 5 α -androst-2-en-17 β -ol on the PE-5 column. Further proof of identity of the two steroids was obtained through GC coelution of the biologically generated material with authentic standard on the PE-5 column. However, during mass spectrometry, the 5 α -androst-2-en-17 β -ol peak value had a shoulder that could not be distinguished from the peak value itself on the basis of the mass spectra. This

shoulder probably represents the corresponding 5 α -androst-2-en-17 α -ol isomer.

Concentrations of 5 α -androst-2-en-17 β -ol during the ovarian cycle

The profiles of pregnanetriol and the luteal phase-specific substance 5 α -androst-2-en-17 β -ol over an 82 week period encompassing five ovarian cycles in an individual female (Aysha) are shown (Fig. 2). The concentrations of pregnanetriol followed a cyclic pattern in which the follicular and luteal phases could be clearly distinguished. On the basis of the time between successive increases in pregnanetriol concentration, the duration of the oestrous cycle was seen to vary from 13 to 15 weeks (mean 14.2 ± 0.5) including a 5–7 week follicular phase with low progestin concentrations and an 8–10 week luteal phase. The FID response of 5 α -androst-2-en-17 β -ol determined by

Fig. 1. Mass spectra of 5 α -androst-2-en-17 β -ol from Asian elephant (*Elephas maximus*) urine (a) compared with synthetic 5 α -androst-2-en-17 β -ol (b), and 5 α -androst-2-en-17-one from Asian elephant urine (c) compared with synthetic 5 α -androst-2-en-17-one (d).

Fig. 3. Profiles of (a) pregnanetriol, (b) 5 α -androst-2-en-17 β -ol and (c) 5 α -androst-2-en-17-one throughout four complete ovarian cycles in an individual female Asian elephant (*Elephas maximus*). Pregnanetriol was measured with an enzyme immunoassay. 5 α -androst-2-en-17 β -ol concentrations were calculated with a standard calibration curve in the range of 10–750 ng ml⁻¹. The equation for the straight line was $y = 0.236 \times 0.015x$ ($r^2 = 0.999$). 5 α -androst-2-en-17-one concentrations were calculated by relating the peak areas of the samples to those of 100 ng 5 α -androst-2-en-17-one ml⁻¹ added to urine. Quantification was obtained with external standards.

gas chromatography showed a similar pattern and increased in a similar way to pregnanetriol ($\rho_s = 0.51$, $n = 163$, $P < 0.01$). Similar profiles for all analytes were obtained for another two cyclic females (data not shown), although these data were restricted to two cycles and samples could be taken only at weekly intervals during the second cycle. On the basis of the FID response, 5 α -androst-2-en-17 β -ol concentrations were positively correlated with those of pregnanetriol ($\rho_s = 0.68$, $n = 40$, $P < 0.01$ and $\rho_s = 0.53$, $n = 30$, $P < 0.01$, respectively).

After 5 α -androst-2-en-17 β -ol had been identified, the detection method was improved, and quantification was obtained with an external standard curve (Fig. 3). Concentrations of up to 225 ng 5 α -androst-2-en-17 β -ol

ml⁻¹ were obtained during the luteal phases, whereas concentrations were < 10 ng ml⁻¹ during follicular periods. As a consequence, the positive association between 5 α -androst-2-en-17 β -ol and pregnanetriol concentrations was improved compared with the positive association obtained by quantifying the FID response ($\rho_s = 0.86$, $n = 97$, $P < 0.01$). The concentrations of the minor compound 5 α -androst-2-en-17-one did not exceed 50 ng ml⁻¹ during the luteal phase and decreased to 5 ng ml⁻¹ during the follicular phase (Fig. 3). Correlations of $\rho_s = 0.85$ ($P < 0.01$) and $\rho_s = 0.97$ ($P < 0.01$) were obtained when 5 α -androst-2-en-17 β -ol was compared with pregnanetriol and 5 α -androst-2-en-17-one, respectively.

One female conceived during the study and the pattern

Fig. 4. Profiles of (a) pregnanetriol and (b) 5 α -androst-2-en-17 β -ol in urine throughout pregnancy in a female Asian elephant (*Elephas maximus*). Week 0 indicates the time of fertile mating. 5 α -androst-2-en-17 β -ol was obtained using the headspace solid-phase microextraction (SPME) technique, with the flame ionization detector (FID) response (area $\times 10^6$) proportional to the concentrations.

of urinary pregnanetriol and 5 α -androst-2-en-17 β -ol during a period of 84 weeks, from 12 weeks before the estimated date of conception (week 0) until 72 weeks of pregnancy is shown (Fig. 4). At the end of a 6 week follicular phase, progesterone concentrations had increased to concentrations of up to 12 $\mu\text{g mg}^{-1}$ creatinine, exceeding those measured during the luteal phase (Fig. 2). Despite considerable variation among samples, the profiles of 5 α -androst-2-en-17 β -ol and pregnanetriol were positively associated ($\rho_s = 0.70$, $n = 77$, $P < 0.01$), with concentrations higher than those seen in the luteal phase.

Use of 5 α -androst-2-en-17 β -ol as a predictor of female reproductive state

Timing of ovulation was estimated retrospectively by comparing the 5 α -androst-2-en-17 β -ol concentrations measured for each of the 15 ovulations that occurred during the study with those deduced from pregnanetriol concentra-

tions. The increased 5 α -androst-2-en-17 β -ol concentrations above a threshold value (mean + 2 SD) during the preceding follicular phase (see data analysis) for 47% of all cycles examined ($n = 15$) indicated that the luteal phase increase occurred on the same date. When the courses of 5 α -androst-2-en-17 β -ol obtained with the SPME method were compared with those from urinary pregnanetriol, cycles with weekly samples ($n = 4$) showed a disagreement in luteal phase increases of not more than 1 week. In cycles in which samples were collected three times per week ($n = 9$), the increase occurred on the same day in three cases, whereas a deviation with a range of -4 to $+5$ days was obtained in the others. In cycles from which daily samples were taken ($n = 2$), the disagreement was 0 and 2 days. The duration of oestrous cycles (or the duration of periods between ovulations) calculated on the basis of the increase in 5 α -androst-2-en-17 β -ol varied from 13.5 to 17.5 weeks (mean 15.1 ± 1.2), and matched those obtained using pregnanetriol analysis (mean 15.2 ± 1.6).

Discussion

Two androgens, 5 α -androst-2-en-17-one and the corresponding alcoholic compound 5 α -androst-2-en-17 β -ol have been identified in the headspace volatile substances of urine of female Asian elephants. The concentrations of 5 α -androst-2-en-17 β -ol and 5 α -androst-2-en-17-one were positively associated with luteal activity and reflected ovarian cyclicity. The results indicate that measurement of 5 α -androst-2-en-17 β -ol should provide a useful additional non-invasive method for monitoring reproductive status in this species.

The present findings of two unsaturated androgens in the elephant with either a 17-oxo- or 17-hydroxy-group were surprising, because 2-en unsaturated 17-oxo and 17-hydroxy androgens have not been demonstrated elsewhere except for a report of 5 α -androst-2-en-17-one in human axillary bacterial isolates (Gower *et al.*, 1997). The demonstration of one keto- and one hydroxy-steroid parallels the situation in pig species in which three steroidal compounds with either a 3-oxo- or a 3-hydroxy-group (5 α -androst-16-en-3-one, 5 α -androst-16-en-3 α -ol and 5 α -androst-16-en-3 β -ol; Patterson, 1968a,b) contribute to the boar pheromone and have been shown to stimulate both sexual behaviour and oxytocin release in females (Mattioli *et al.*, 1986; Claus and Schams, 1990). The same 16-en unsaturated androgens have also been demonstrated in men (Claus and Alsing, 1976), male moose (*Alces alces*; Claus 1994) and male camels (*Camelus dromedarius*; Claus *et al.*, 1999). So far, the role of pheromones has been attributed to these steroids only in pigs, in which they trigger an immobilization (lordosis)-like behaviour in females.

The origin and possible functions of 5 α -androst-2-en-17-one and 5 α -androst-2-en-17 β -ol in Asian elephants are unknown. The strong association of periods of increased urinary 5 α -androst-2-en-17-one and 5 α -androst-2-en-17 β -ol secretion with the luteal phase of the cycle indicate that these androgens may be gestagen metabolites. However, this conclusion seems unlikely in view of the results of metabolism studies in several species (Schwarzenberger *et al.*, 1997a,b) and of GC-MS analysis of urinary gestagen metabolites of Asian elephants (Niemüller *et al.*, 1993), which indicate that gestagens are metabolized to hydroxylated pregnanes. Alternatively, 5 α -androst-2-en-17-one and 5 α -androst-2-en-17 β -ol might both be androgen metabolites. Gestagens are precursors for androgen biosynthesis and high quantities of conjugated androgens have been reported in the urine of men (Mareck-Engelke *et al.*, 1995a; Choi *et al.*, 2000) and women (Mareck-Engelke *et al.*, 1995b). Urinary 3 α ,17 β -androstane-3 α -diol is a useful indicator of androgenic activity in men (Deslypere *et al.*, 1982) and has also been used to evaluate circannual androgenic activity in the male Eld's deer (*Cervus eldii thamin*), an endangered ungulate species (Monfort *et al.*, 1995). However, to our knowledge, no previous study has demonstrated luteal-dependent androgen secretion in females of any mammal species. Moreover, measurements

of immunoreactive testosterone in female elephant urine did not reveal luteal phase increases (M. Heistermann and M. Dehnhard, unpublished). Thus, it is unlikely that the patterns of urinary 5 α -androst-2-en-17-one and 5 α -androst-2-en-17 β -ol excretion described here in Asian elephants reflect the secretion of hormonally active androgens (for example, testosterone).

The presence of substantial amounts of 5 α -androst-2-en-17-one and 5 α -androst-2-en-17 β -ol in the urine of female elephants indicates that these androgens are not merely metabolic by-products. Thus, we hypothesize a route of biosynthesis of 2-unsaturated androgens in the ovary of Asian elephants that deviates from the established routes of androgen and oestrogen biosynthesis (precursors: androst-4-en,3,17-dione and androst-5-en,3 β ,17 β -diol, respectively). Such biosynthetic pathways have been described for the testicular synthesis of 16-androstenes (5 α -androst-16-en-3-one, 5 α -androst-16-en-3 α -ol and 5 α -androst-16-en-3 β -ol) in pigs (Gower, 1972) and men (Claus and Alsing, 1976). In both cases, 5,16-androstadien-3 β -ol and 4,16-androstadien-3-one have been shown to be important precursors of 16-androstenes (Gower and Ruparelia, 1993) and ovarian production has been suggested for urinary 5 α -androst-16-en-3 α -ol in women (Brooksbank, 1962).

The function of 5 α -androst-2-en-17-one and 5 α -androst-2-en-17 β -ol in Asian elephants is unclear, but it is possible that it is part of a multidirectional chemical communication system. Although a possible role as pheromones has yet to be demonstrated, both 2-unsaturated androgens might serve as pheromones to influence ovarian function in members of a group. For example, longitudinal data from pregnant triol analyses revealed that females with close social relationships tend to synchronize their timing of oestrus (Oerke *et al.*, 1999), and it is possible that pheromones play an important role in this process. In addition, changes in 5 α -androst-2-en-17-one and 5 α -androst-2-en-17 β -ol concentrations in urine might inform female members of the group about the reproductive state of an individual and may also serve as a signal of reproductive condition to males. Further studies to clarify the biological function and pheromonal activity of these substances are required.

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