PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 84 (2003) 383–391

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Headspace solid-phase microextraction (SPME) and gas chromatography–mass spectrometry (GC–MS) for the determination of 5α-androst-2-en-17-one and -17β-ol in the female Asian elephant: application for reproductive monitoring and prediction of parturition☆

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Abstract

Asian elephants are not self-sustaining in captivity. The main reasons for this phenomenon are a low birth rate, an aging population, and poor calf-rearing. Therefore, it is essential that reproductive rates had to be improved and there is need for rapid quantitative measures to monitor reproductive functions focussing on estrous detection and the prediction of the period of parturition. The objective of this study was to develop a method which combines headspace solid-phase microextraction (SPME) and gas chromatography–mass spectrometry (GC–MS) for analyses of 5α-androst-2-en-17β-ol and -17-one to prognose estrous and to predict the period of parturition. SPME was carried out with a CTC Combi Pal system.

The course of the luteal phase-specific substance 5α-androst-2-en-17β-ol and -17-one followed a cyclic pattern in which the follicular and luteal phases could be clearly distinguished (mean estrous cycle length, 15 ± 1.4 weeks). Based on daily urine samples, estrous prognosis might be possibly based on the initial 5α-androst-2-en-17β-o1 increase at the end of the follicular phase. Parturition prognosis was performed in three elephant cows based on the 5α-androst-2-en-17β-o1 drop to baseline levels 5–4 days prior parturition. Experiments revealed that 5α-androst-3α-ol-17-one and probably 5α-androst-3α-ol-17β-ol are generated from sulfate conjugates by a thermal process.

Keywords: Elephant; Reproduction; Estrous; Parturition; Headspace analysis; 2-En unsaturated steroids

1. Introduction

Environmental disturbance is a major cause of population decline in endangered species [1]. This concerns in particular the Asian and African elephants. The African elephants, *Loxodonta africana* (like the Asian relative, *Elephas maximus*) is listed in Appendix 1 of CITES as an endangered species. African elephant populations were heavily poached and, thus dramatically reduced in the late 1970s and early 1980s [2]. As a result of the successful international ivory trade ban African elephant populations have stabilized and even increased to a population of around 600,000 [3]. The Asian species once occupied vast areas of Asia. Its remaining population of around 50,000 individuals is now restricted mainly to forested areas of the Indian subcontinent and Southeast Asia that have not yet been taken over by human settlements [4].

In captivity, both African and Asian elephant populations are not self-sustaining because of a low birth rate, an aging population and poor calf-rearing. First year mortality for the Asian elephant is approximately 40%. Data from captive African elephants indicate that they have also reproduced poorly and experienced low juvenile survival in the zoos. Without a distinct increase in reproductive rate, both captive elephant populations will drop and include the risk

☆ Poster paper presented at the 15th International Symposium of the Journal of Steroid Biochemistry and Molecular Biology. "Recent Advances in Steroid Biochemistry and Molecular Biology", Munich, Germany, 17–20 May 2002.

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0960-0760/03/$ – see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0960-0760(03)00056-6

to be demographically extinct within several decades [4,5]. To improve management activities directed on reproduction it is necessary to monitor reproductive functions as estrous detection and the prediction of the period of parturition.

Reproductive monitoring in elephants is traditionally performed by immunoreactive progesterone (P_4) or 5α-reduced gestagen measurements in blood [6–10]. Based on profiles generated by these studies, an ovarian cycle length of 13–17 weeks has been established for both species, consisting of a 4–6 weeks follicular phase and a 9–11 weeks luteal phase [11]. More recently, alternative non-invasive techniques based on the analysis of urinary and fecal progestin metabolites have also been developed [9,12–14]. A further alternative approach to provide information on female reproductive status in the Asian elephant might be the measurement of pheromones. Pheromones are olfactory stimuli which release specific behavioral or endocrine reactions in the recipient [15]. Behavioral effects of estrous-related pheromones on males have been described in several species, including mouse [16–18], hamster [19], sheep [20,21], cow [22], cotton-top tamarin [23] and the Asian elephant [24], but the chemical nature of the substances involved is mostly unknown. In the Asian elephant an estrous-related pheromone, (*Z*)-7-dodecenyl acetate has been identified. It is secreted with the urine during the follicular phase, elicits a chemosensory response, the Flehmen reaction in elephant bulls [24]. However, there is no distinct pre-ovulatory increase of (*Z*)-7-dodecenyl acetate which can be used to predict estrous [25]. There is also evidence for the existence of gestagen dependent pheromones in several vertebrate taxa, including fish, reptiles and mammals. In fish, pheromonally active estrogen and gestagen metabolites are released into the water [26,27]. Exposure to the pheromone 17α-20β-dihydroxy-4-pregnen-3-one enhances the behavioral spawning success, sperm production and sperm motility of male goldfish [28]. In the rabbit, progesterone administration effectively reinstates the emission of the nipple pheromone in ovariectomized animals [29]. These examples support the idea that gestagen-dependent substances serving as chemical signals may also exist in elephants. It was our initial aim to investigate the presence of estrous cycle-related volatiles in the urine of Asian elephants which might be available as chemosignals for elephants. In a previous report, we demonstrated the presence of two luteal phase specific steroidal volatile compounds (5α-androst-2-en-17β-ol and -17-one) in elephant urine [30]. Their quantification with headspace solid-phase microextraction (SPME) and analysis by gas chromatography revealed that they were positively correlated with the concentration of urinary pregnanetriol an abundant urinary progesterone metabolite in the Asian elephant and gestagens in blood plasma [31]. So far, one of the substances, 5α-androst-2-en-17-one has been demonstrated in human axillary bacterial isolates [32]. In a study of Labows et al. [33] on human apocrine secretions, the production of 5α-androst-2-en-17-one from androsterone sulfate by a thermal process has been shown. In the porcine species, three steroidal compounds with either a 3-oxo- or 3-hydroxy-group (5α-androst-16-en-3-one, 5α-androst-16-en-3α-ol and -3β-ol) contribute to the boar pheromone [34,35] and have been shown to stimulate both an immobilization-like behavior and oxytocin release in sows [36,37]. More recently, the same 16-en unsaturated androgens have also been demonstrated in male moose (*Alces alces* [38]), male camel (*Camelus dromedarius* [39]), in blood plasma [40] and axiallary secretions of man [41] and in the urine of woman [42].

The objective of this study was: (1) to develop a method which combines headspace solid-phase microextraction (SPME) and gas chromatography–mass spectrometry (GC–MS) for highly sensitive and specific analyses of 5α-androst-2-en-17β-ol and -17-one; (2) to investigate the use of 5α-androst-2-en-17β-ol and -17-one analyses for prognosis of ovulation; (3) to predict the period of parturition in the Asian elephant based on 2-unsaturated C_{19}-steroid analysis; and (4) to perform experiments to explain the urinary origin of both the 2-unsaturated C_{19}-steroids.

2. Material and methods

2.1. Animals and sample collection

Seven adult female Asian elephants (ages from 14 to 31 years) were involved in the study. They 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 prior to the study. From 1997 to 2001, urine samples were collected 1–3 times a week during the follicular and luteal phase. They were obtained from a total of 15 non-conception cycles and three conception cycles including pregnancy. Several weeks prior the date of the assumed parturition sampling frequency was increased to daily urine samples. In one of the animals, daily urine samples were collected from a follicular phase. 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 volatiles. All the samples were frozen immediately after collection and stored at −20 °C until analyzed. Freezing at −20 °C for 4 months and incubation (4 h at 37 °C and 72 h at room temperature) did not result in a significant loss of 2-unsaturated C_{19}-steroids. In two cases where parturition prognosis were performed urine samples were send by regular postage and reached the lab within 1–2 days.

3. Determination of female reproductive status

The date of ovulation was determined by a rise in urinary 5α-androst-2-en-17β-ol levels above a given threshold

value (mean+2S.D.) of the preceding follicular phase values [9].

4. SPME sampling

4.1. Gas chromatography

SPME sampling and gas chromatographic analyses of 5α-androst-2-en-17β-ol and -17-one were carried out manually as described in a previous study [30] using a fiber with a 85 μm polyacrylate coating (Supelco, Bellefonte, PA, USA). The vial was then thermostatically heated to 110 °C and agitated with a magnetic stirring bar and the fiber was exposed into the headspace above the surface of the urine (60 min). The analytes were desorbed in the injector of the gas chromatograph at 300 °C, desorption was carried out in the splitless mode, and the fiber was maintained for approximately 15 min in the chromatograph injector.

Analyses were carried out in a Perkin-Elmer (PE) AutoSystem XL gas chromatograph provided with a flame ionization detector (FID). A 30 m × 0.25 mm i.d. (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 290 °C at 10 °C min^{-1}. The carrier gas was nitrogen at 140 kPa. The detector was kept at 300 °C.

4.2. Gas chromatography–mass spectrometry (GC–MS)

SPME was carried out with a CTC Combi Pal system autoinjector at 110 °C for 60 min using a fibre with a 85 μm polyacrylate coating. An aliquot of 4.5 ml urine, 0.5 ml 2 M Tris buffer (Merck, Darmstadt, Germany, pH 7.0), 1.83 g NaCl (Merck, Darmstadt, Germany), and 500 ng 5α-androst-3-one (Sigma, Deisenhofen, Germany) as internal standard were added into 20 ml headspace vials (Perkin-Elmer, Norwalk, CT, USA). The GC–MS determinations were conducted with a Shimadzu GCMS-QP 5050. The MS acquisition was performed in SIM, by monitoring the ions *m/z* 218 for 5α-androst-2-en-17-one and *m/z* 220 for 5α-androst-2-en-17β-ol. 5α-Androst-3-one was used as an internal standard added to urine prior analyses (100 ng ml^{-1}) and monitored at *m/z* 202. The samples were analyzed using a 50 m SE-54 capillary column (0.32 i.d. and 1 μm film thickness, CS, Langerwehe, Germany). Ultrapure helium was used as carrier gas, with a column head pressure setting of 41.2 kPa. Injector temperature was 300 °C; the interface temperature was maintained at 300 °C and ionizing voltage was at 1.2 kV. Splitless injection mode was used, the purge valve was turned on 15 min after injection, with a split flow of 8 ml min^{-1} during the GC run. The GC oven was kept at 50 °C for 2 min and increased to 300 °C at 50 °C min^{-1}. The mass spectra were identified by computer MS library research and compared with those of the authentic standards.

5. Quantification

5.1. Gas chromatography

Quantification was carried as described in a previous study [30] using a calibration line by adding various amounts of 5α-androst-2-en-17β-ol (Steraloids, Newport, RI, USA; 10, 25, 50, 75, 100, 250, 500, and 750 ng) and a constant amount of 100 ng ml^{-1} 5α-androstan to blank urines from the follicular phase. 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}.

5.2. Gas chromatography–mass spectrometry (GC–MS)

A calibration line was prepared by adding various amounts of 5α-androst-2-en-17β-ol and -17-one (both obtained from Steraloids, Newport, RI, USA; 2.5, 5, 10, 25, 50, 75, 100, 200, and 400 ng ml^{-1}) and a constant amount of 100 ng ml^{-1} 5α-androst-3-one (internal standard) to blank urines obtained from an elephant cow with hormonal contraception [32] that contained no detectable amounts of the analytes. The peak areas of the compounds were quantitatively integrated. A calibration curve for the relative peak area of 5α-androst-2-en-17β-ol and -17-one to the internal standard (5α-androst-3-one) versus the concentration of the 2-en-steroids were obtained. The calibration curve of 5α-androst-2-en-17-one showed a good linearity in the range of 2.5–200 ng ml^{-1}. The equation for the straight line was $y = 0.01–0.006x$ ($r^2 = 0.999$) and $y = 0.41–0.033x$ ($r^2 = 0.979$) for 5α-androst-2-en-17β-ol and -17-one, respectively. Urinary concentrations were calculated from the peak ratios according to the equation.

The detection limit for the determination of 5α-androst-2-en-17β-ol and -17-one, at a signal-to-noise ratio of 2:1, was found to be 1 ng ml^{-1} and approximately 0.5 ng ml^{-1}, respectively. To compare previous GC measurements with new GC–MS data, 4–6 samples of four elephant cows were measured with both the analytical methods. The correlations between the concentrations varied individually and ranged from $r = 0.98$ to 0.87. For one animal (as shown in Fig. 3) previous GC data (weeks, 1–159) were adapted to the recent GC–MS data set (from weeks 160) by dividing the previous data with 12.5.

6. Incubation experiments (GC–MS)

To explain the urinary origin of the 2-unsaturated C_{19}-steroids, three series of incubation experiments were carried out over 60 min at 70, 80, 90, 100 and 110 °C. To all urines, the internal standard 5α-androst-3-one (100 ng ml^{-1}) was added. Experiments were carried out with: (1) pooled urines from a pregnant animal containing high amounts of endogenous 2-unsaturated C_{19}-steroids; (2) blank urine

spiked with 5a-androst-3α-ol-17-one sulfate (500 ng ml^{-1}); and (3) blank urine spiked with 5α-androst-2-en-17β-ol and -17-one (100 ng ml^{-1}, each).

7. Data analysis

The date of ovulation was determined by a rise in urinary 5α-androst-2-en-17β-ol levels above a given threshold value (mean + 2S.D.) of the preceding follicular phase values as described for urinary gestagen metabolites [9].

8. Results

8.1. Concentrations of 5α-androst-2-en-17β-ol and -17-one during the ovarian cycle

The course of the luteal phase-specific substance 5α-androst-2-en-17β-ol and -17-one measured by GC–MS over a 28-weeks period is shown in Fig. 1. The concentrations followed a cyclic pattern in which the follicular and luteal phases could be clearly distinguished. During the luteal phases, the concentrations of 5α-androst-2-en-17β-ol and -17-one reached 91 and 14 ng ml^{-1}, respectively. Similar profiles for both the analytes were obtained from other cycling females (data not shown). Due to the higher concentrations of 5α-androst-2-en-17β-ol compared to -17-one, further monitoring of luteal activity was based on -17β-ol measurements.

To evaluate the course of 5α-androst-2-en-17β-ol and -17-one prior to estrous, daily urine samples were collected during two follicular phases in one animal (Fig. 2). Both the steroids steadily dropped down to zero. 5α-Androst-2-en-17β-ol was measured in low concentrations within the first weeks of the follicular phase reaching zero levels at the end of the follicular phase. A few days prior ovulation the concentrations increased slightly followed by a transitory decrease (assumed time of ovulation, see Section 11) just before a rapid increase afterwards.

8.2. Concentrations of 5α-androst-2-en-17β-ol and -17-one during pregnancy

Fig. 3 shows the pattern of urinary 5α-androst-2-en-17β-ol measured by GC and GC–MS during a period of 198 weeks, from 12 weeks prior to the estimated date of conception (week 0) until the end of pregnancy (weeks 94). The calf was hand-reared and died 8 months after birth (weeks 134). The elephant cow reassumed cyclic ovarian activity with increasing luteal amplitudes approximately 24 weeks after parturition. The detailed peripartal course is shown as an enlargement in Fig. 3. As the end of pregnancy approached concentrations decreased continuously over several weeks followed by a drop to baseline levels (concentrations of an elephant cow with hormonal contraception). This drop was used as an indicator that parturition is imminent, which took place 4 days later. This type of parturition prognosis was confirmed in two additional cows (Fig. 4). In both the animals a dramatic drop occurred during the last 10 days before parturition and baseline levels were measured 5–4 days prior parturition.

9. Origin of urinary 5α-androst-2-en-17-one

To explain the urinary origin of the 2-unsaturated C_{19}-steroids, three series of incubation experiments were

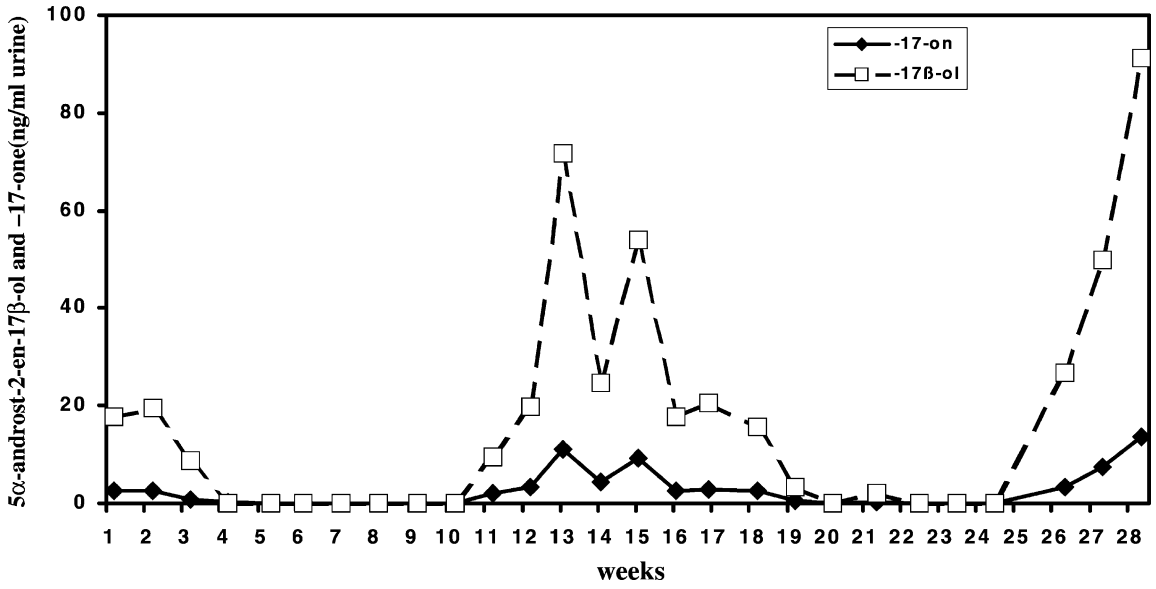

Fig. 1. Individual course of the luteal phase-specific substances 5α-androst-2-en-17β-ol and -17-one measured by GC–MS over an 28-weeks period in one cycling Asian elephant.

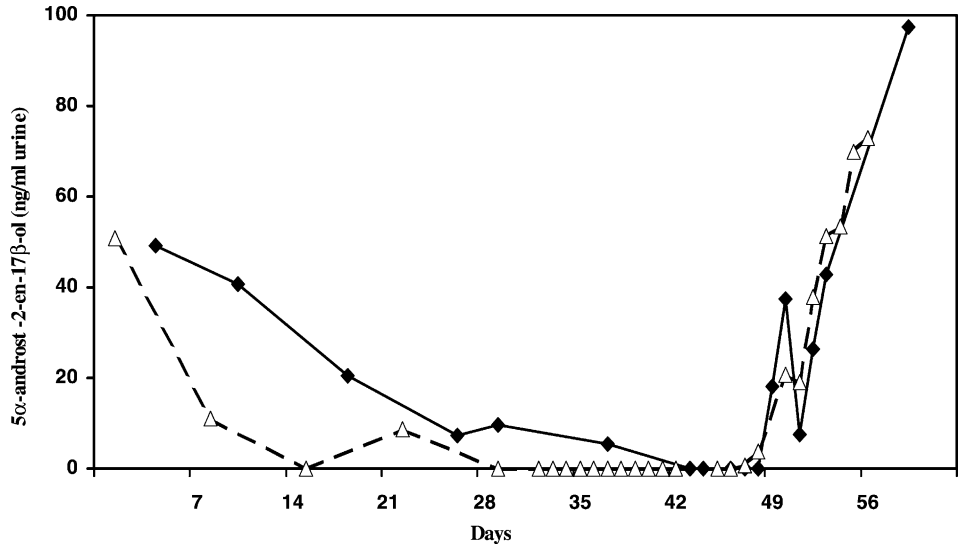

Fig. 2. Course of 5α-androst-2-en-17β-ol during two follicular phases in one Asian elephant cow based on frequent urine sampling.

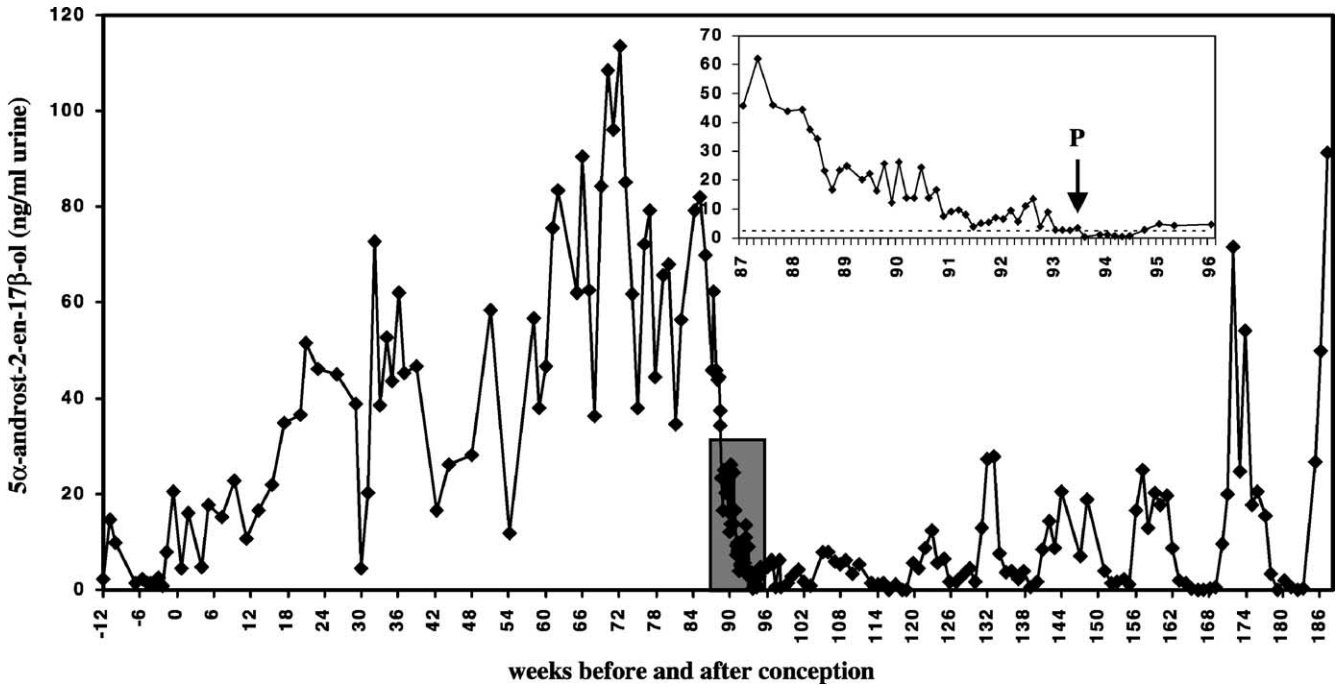

Fig. 3. Course of urinary 5α-androst-2-en-17β-ol concentrations in a pregnant animal from 12 weeks prior to the estimated date of conception (week 0) until the end of pregnancy (weeks 94). Eight months after birth (P, weeks 134) the calf died. The detailed peripartal course (weeks 87–96) is shown as enlargement.

carried out at temperatures between 70 and 110 °C. Maximal recovery of 5α-androst-2-en-17β-ol and -17-one added to urine was achieved at 90 °C for both the androgens (Fig. 5a). Even higher temperatures did not led to an increased recovery. However, when urine from a pregnant animal which contains high amounts of endogenous 5α-androst-2-en-17β-ol and -17-one was investigated, maximal recovery was obtained at 110 °C (Fig. 5b). This discrepancy led to the assumption, that endogenous 5α-androst-2-en-17β-ol and -17-one probably might be generated from precursors by a thermal process. This hypothesis was confirmed for 5α-androst-2-en-17-one after addition of androsterone sulfate to urine. Maximal recovery of 5α-androst-2-en-17-one was obtained at 110 °C (Fig. 5c) which fits with the thermokinetic course of endogenous 5α-androst-2-en-17-one. This confirmed our presumption that 5α-androst-2-en-17-one might be generated from androsterone sulfate by a thermal process. According to

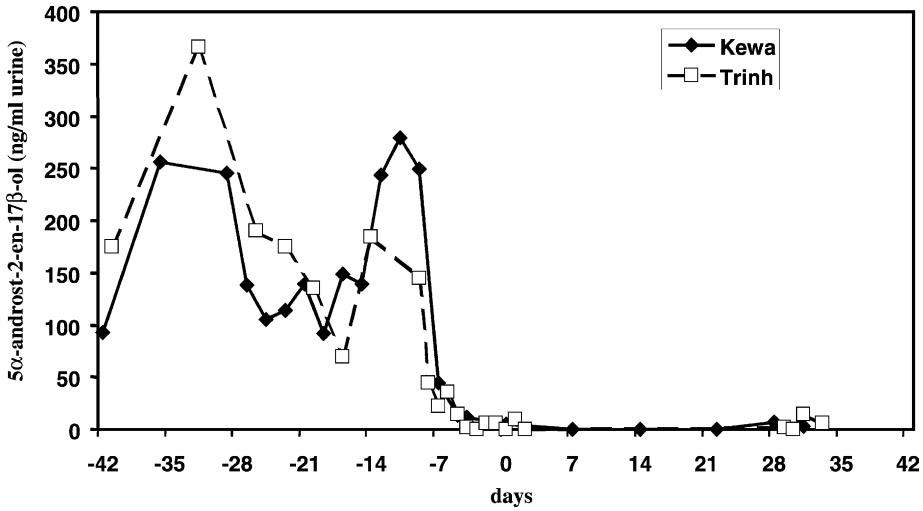

Fig. 4. Peripartal course of urinary 5α-androst-2-en-17β-ol concentrations in two additional elephants cows. Day 0 corresponds to the day of parturition.

the mass spectra obtained, 5α-androst-2-en-17-one could be clearly identified (Fig. 5c). The mass spectra is similar to those of the synthetic compound added to urine (Fig. 5a) and endogenous 5α-androst-2-en-17-one (Fig. 5b). 5α-Androst-2-en-17-one showed base peaks at *m*/*z* 218, 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 [33].

10. Application to other species

Both 2-en unsaturated steroids were detectable in urines obtained from one cycling African elephant cow. However, luteal concentrations were approximately one-tenth of those reached in the Asian elephant species. When different urine samples from zoo animal species were screened for 2-en unsaturated steroids, 5α-androst-2-en-17-one was clearly detected in a urine sample of a male Takin (*Budorcas taxicolor tibetana*).

Comparative analyses of volatiles in human revealed high amounts of 2-en unsaturated androgens in adult males and females. In addition, pre-puberal girls revealed diverging amounts of these substances, suggesting an adrenal synthesis in human.

11. Discussion

In a previous study, we have identified two androgens, 5α-androst-2en-17-one and the corresponding alcoholic compound 5α-androst-2-en-17β-ol, in the headspace volatiles of urine of female Asian elephants [30]. The concentrations of 5α-androst-2-en-17β-ol and -17-one were positively associated with luteal activity and reflected ovarian cyclicity. Specificity and sensitivity was increased in a newly established GC–MS method when MS acquisition was performed in SIM, by monitoring the ions *m*/*z* 218 for 5α-androst-2-en-17-one and *m*/*z* 220 for -17β-ol. This was confirmed with urine samples from an acyclic cow due to hormonal contraception [31]. Whereas, previous GC measurements revealed a noise signal at the retention time of 5α-androst-2-en-17β-ol, MS acquisition in SIM did not detect a *m*/*z* 220 signal.

In combination with a headspace system autoinjector this method is an useful additional non-invasive method for monitoring reproductive status in elephants and other species. The treatment of elephant urine can be performed within 1.5 h. Therefore, this is a rapid method for detection of cycle stage, for prognosis of parturition and probably for prognosis of estrous. Successful prognosis of parturition based on GC measurements was shown in one animal (Fig. 1). The 5α-androst-2-en-17β-ol concentrations continuously decreased over a period of several weeks prior parturition lineally approaching the levels of the contracepted elephant cow. A final drop to non-luteal (baseline) levels was used as an indicator that parturition is imminent. In two other animals, a rapid and distinct drop occurred during the last 10 days before parturition and baseline levels were measured 5–4 days prior parturition. This type of course simplified parturition prognosis which was pronounced 1 day before. The different patterns of luteolytic activity at the end of gestation reflect individuality. Secretion is variable, however, making the selection of a low cut-off value difficult. Both, dramatic pre-partum decreases of luteal activity and extremely low baseline approaching levels during the last trimester have been reported [43,44]. Therefore, the daily monitoring of luteal activity during the last weeks of gestation is highly recommended.

In many species timing of parturition is initiated by the fetus through activation of its hypothalamic–pituitary–adrenal axis and a corresponding increase in cortisol. As predictor of parturition, however, the measurement of urinary

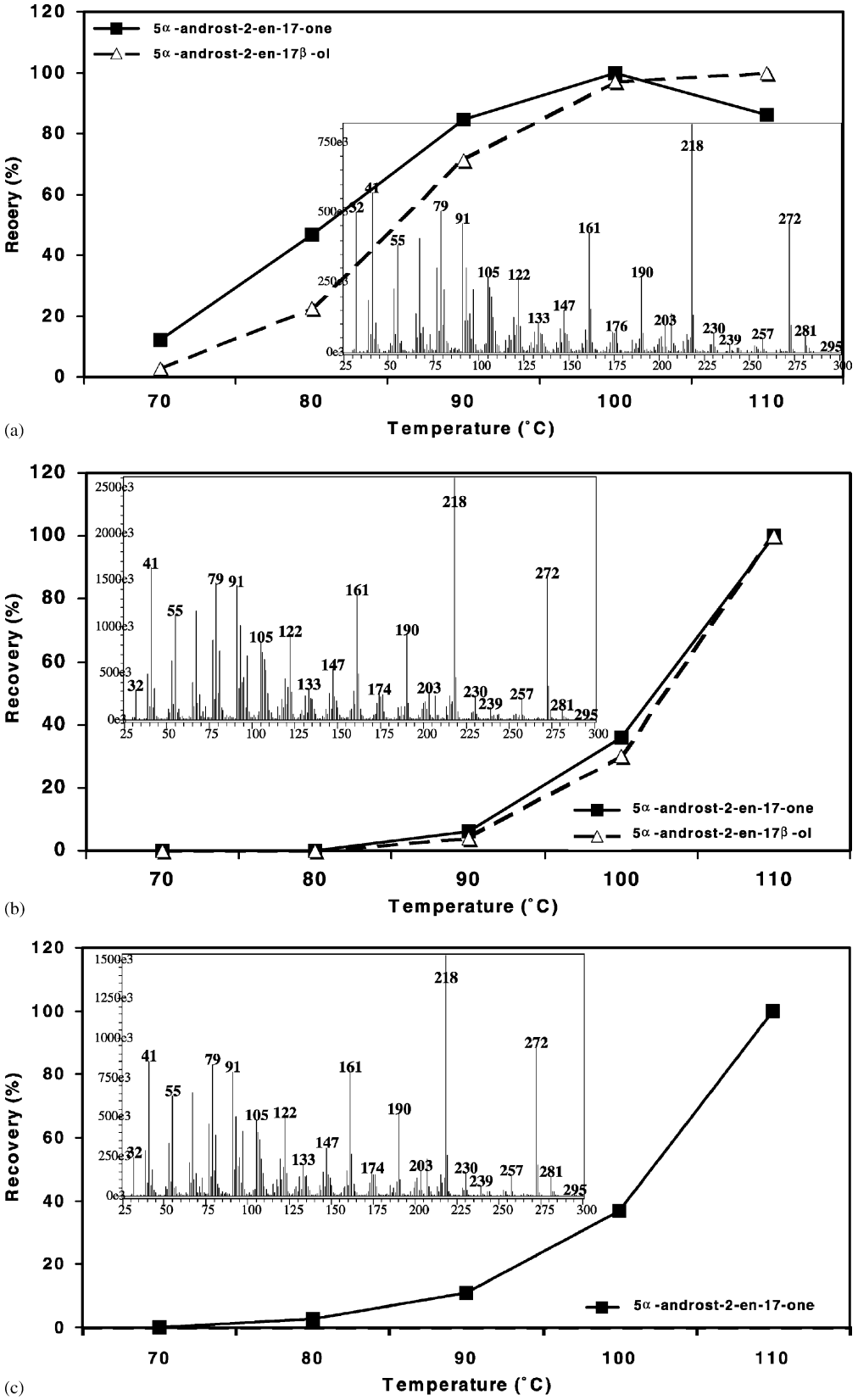

Fig. 5. Origin of 2-unsaturated C_{19}-steroids in the urine of the Asian elephant. (a) Blank urine spiked with 5α-androst-2-en-17β-ol and -17-one, (b) urine from late pregnancy containing high amounts of both steroids, and (c) blank urine spiked with androsterone sulfate were heated (for 60 min) at temperatures between 70 and 110 °C. Maximal recovery was set to 100% and mass spectra of 5α-androst-2-en-17-one (inserts) were recorded from those samples which were heated at 110 °C.

cortisol metabolites is not likely to be useful because maximal concentrations due to adrenal activation were obtained in response to the stress of parturition on the day after birth [43,45].

We found some indications, that monitoring 5α-androst-2-en-17β-ol levels in urine might be useful for estrous prognosis in elephants. A few days prior to ovulation, the concentrations increased slightly followed by a transitory decrease (assumed time of ovulation [43]) just before a rapid increase afterwards (Fig. 2). Before this very important application of GC–MS can be recommend further investigations on more cycles and elephants should be performed. Simultaneously determination of serum LH will be helpful to correlate the 5α-androst-2-en-17β-ol increase to ovulation.

Possible functions of 5α-androst-2-en-17-one and -17β-ol in the Asian elephant are still unknown. We could show that they were generated from precursors by a thermal process (proven for 5α-androst-3α-ol-17-one from androsterone sulfate, Fig. 5). The presence of substantial amounts of 5α-androst-2-en-17-one and -17β-ol conjugates in the urine of female elephants suggests that they are metabolic by-products. This argues against our previous hypothesis that the volatile androgens may have a communicatory function and may act as pheromones in the Asian elephants [30].

We suggest, that there is a distinct possibility that 5α-androst-2-en-17-one and -17β-ol conjugates might serve as pheromones in elephants. The Flehmen sponse is an integral part of pheromone cueing in elephants. Bulls investigate the reproductive status of female elephants in urine using the Flehmen reaction. During the Flehmen response, liquids containing compounds are transported into the vomeronasal organ [24], which also enables the animal to investigate non-volatile urinary compounds [46]. Changes in 5α-androst-2-en-17-one and -17β-ol levels in urine might inform female members of the group about the reproductive status 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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