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Monitoring ovarian cycle and pregnancy in the giant anteater (*Myrmecophaga tridactyla*) by faecal progestagen and oestrogen analysis

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Abstract

Oestrogen and progestagen metabolites were measured in the faeces of five female giant anteaters (*Myrmecophaga tridactyla*), to characterise the oestrous cycle and pregnancy. Faecal samples were collected twice weekly for a minimum of 6 months, and immunoreactive progestagens and oestrogens were analysed using enyzme immunoassays (EIA). For progestagens, two antibodies that cross-reacted with 20α-hydroxy- or 20-oxo-progestagens were used. Both assays effectively monitored ovarian cyclicity; however, the concentrations obtained using the antibody for 20α-hydroxy-progestagens were higher, and the hormonal changes were more pronounced. Regular ovarian cycles were identified in three of the five females. Average (±SEM) length of the oestrous cycle ($n = 10$) was 51.4 ± 5.6 days. Peak concentrations of 20α-hydroxy-progestagens ranged from 80–660 ng/g of faeces and those of oestrogens from 20–100 ng/g. Hormone concentrations were measured during parts of two pregnancies and during four post-partum periods. The length of one gestation (from oestrous oestrogen peak until parturition) was 184 days. In the second half of gestation, progestagen concentration started to increase above luteal phase values; in the week before parturition it was ~20 times higher than those during the luteal phase. Concentrations of excreted oestrogens began to increase after two thirds of gestation and exceeded that of the follicular phase by ~2.5-fold in the week before parturition. Onset of ovarian cyclicity

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after parturition varied from 4–11 weeks. In conclusion, the measurement of faecal immunoreactive progestagens and oestrogens in the giant anteater indicated an ovarian cycle of ~7 weeks in length and provided potentially useful data for successful breeding management.

Keywords: Non-invasive; Ovarian function; Pregnancy diagnosis; Xenarthra; Progesterone metabolites

1. Introduction

The giant anteater (*Myrmecophaga tridactyla*) is a member of the family of Myrmecophagidae belonging to the order Xenarthra, which includes armadillos (*Dasypodidae*) and sloths (*Bradypodidae* and *Choloepidae*). Giant anteaters are distributed throughout Central and South America as far as northern Argentina and occupy nearly all biotopes from rainforests to savannahs (Moeller, 1988). They are endemic mainly in the Cerrado savannah, an area being rapidly transformed into plantations (Bartmann, 1994); therefore, the number of giant anteaters in the wild is decreasing. They have been exhibited in zoos since the mid-1800s, but successful breeding is rare. According to the International Studbook for the giant anteater (Bartmann, 1978–1994), an average of only eight births per year were registered in zoos world-wide, and only about half of the new-borns survived. Loss of new-borns is caused by stillbirth, incorrect maternal behaviour or quite often the males injure or kill the infants immediately after birth. As females do not exhibit discernible specific behaviour during oestrus, females and males are kept together to create good conditions for breeding. But they have to be divided before birth, which is difficult because pregnancy is not recognised in many cases and mating occurs even some days before delivery (Bartmann, 1983, 1994). Much is known about behaviour and hand-rearing of young anteaters (Bartmann, 1983; Poglayen-Neuwall, 1990), but little concerning reproductive endocrinology. The giant anteater is a non-seasonal breeder, giving birth at all times of the year both in captivity and in the wild (Bartmann, 1994). Gestation lasts 180–190 days (Bartmann, 1983; Moeller, 1988; Poglayen-Neuwall, 1990).

Information about reproductive steroid hormones are only available for the lesser anteater (*Tamandua tetradactyla*). Hay et al. (1994) measured pregnanediol-glucuronide and conjugated oestrogens in urine samples from two non-pregnant females in conjunction with monitoring of changes in vaginal cytology. They observed regular oestrous cycles of ~42 days in one of the animals with no apparent effect of season.

For many other mammalian species, reproductive status has been assessed by monitoring the excretion of ovarian steroid metabolites (progestagens and oestrogens) in faeces (Schwarzenberger et al., 1996). Progestagens can be grouped according to their cross-reactivity with group-specific antibodies into pregnanes having a 20-oxo, a 20α- or a 20β-hydroxyl group (Schwarzenberger et al., 1997). The aim of the present study was to demonstrate that ovarian cycles, pregnancy and the post-partum period can be characterised in the giant anteater by measuring faecal immunoreactive oestrogens and progestagens.

2. Materials and methods

2.1. Animals

Three of the five female anteaters used in this study were housed at the zoo Tierpark Dortmund, Germany (Studbook number: 0035, 0245, 0255) and the other two at the zoo Tierpark Hellabrunn, Munich, Germany (Studbook number: 0067, 0069). The animals 0035, 0067 and 0245 were wild-borne, and at the beginning of the study they were 13, >8 and >2 years of age, respectively. Whereas the females 0069 and 0255 were borne in zoos, they were 7 and 2 years of age. Depending on climate and weather conditions, the animals were kept in heated indoor facilities with controlled access to outdoor enclosures. Females and males were housed together during daytime and separated over night. They were fed with a diet consisting of a mixture of evaporated milk, minced meat, dog food, cereals, fruits and a vitamin–mineral supplement.

2.2. Sample collection

Faecal samples were collected twice weekly from August through early February from all five animals, which were non-pregnant at the beginning of the sampling period. Subsequently, samples from anteaters at the Tierpark Dortmund zoo were collected intermittently for a 2-year period because of expected gestation ($n=2$) or after parturition ($n=4$). In detail, female 0035: from Days 10–104 post-partum and for a period of 81 days due to supposed gestation; female 0245: two post-partum periods from Days 9–147 and from Days 2–95; female 0255: from Day 150 of pregnancy until Day 44 post-partum. Faecal samples were stored at -20°C until analysis.

2.3. Extraction and analysis

For extraction of oestrogens and progestagens, faeces (0.5 g) were vortexed for 30 min in distilled water (0.5 ml) and methanol (4 ml). Petroleum-ether (3 ml) was added and the tube was re-vortexed for 15 s to remove apolar lipids. After centrifugation, an aliquot of the methanol fraction was diluted with assay buffer (20 mmol Trishydroxyaminomethan, 0.3 mol NaCl, 0.1% bovine serumalbumin and 0.1% Tween 80; pH 7.5 with 1 mol HCl) for EIA analysis (Schwarzenberger et al., 1991).

The EIA for unconjugated total oestrogens was performed as reported by Palme and Möstl (1994) using an antibody against 17β-oestradiol-17-HS:BSA and oestrone as standard. Cross-reactivities for this assay were: oestriol (129%), oestrone (100%), oestradiol-17β (70%) and oestradiol-17α (19%). The intra- and interassay coefficients of variation for a pool of faecal samples were 9.4% and 12.3%, respectively.

The two assays for analysing the faecal progestagen metabolites have been described by Schwarzenberger et al. (1993). One antibody was raised against 4-pregnene-6α-ol-3,20-dione-6-HS:BSA and cross-reacted mainly with 20-oxo-progestagens (20-oxo-P). The intra- and interassay coefficients of variation were 11.5% and 14.3%, respectively. The antibody showed the following cross-reactivities: 4-pregnene-3,20-dione (100%), 5β-pregnane-3,20-dione (71%), 5α-pregnane-3,20-dione (40%), 5-pregnene-3β-ol-20-

one (28.6%), 5α-pregnane-3β-ol-20-one (18.2%), 5β-pregnane-3β-ol-20-one (6.5%), 4-pregnene-20α-ol-3-one (6.3%) and 5β-pregnane-3α-ol-20-one (3.3%). The other antibody was raised against 5β-pregnane-3α, 20α-diol-3-glucuronide:ovalbumin and cross-reacted with the following 20α-hydroxy-progestagens (20α-OH-P): 5β-pregnane-20α-ol-3-one (176%), 4-pregnene-20α-ol-3-one (150%), 5β-pregnane-3α, 20α-diol (100%), 5β-pregnane-3β, 20α-diol (100%), 5α-pregnane-3β, 20α-diol (55.6%), 5α-pregnane-20α-ol-3-one (50.0%), 5α-pregnane-3α, 20α-diol (24.0%) and 5-pregnene-3β, 20α-diol (16.6%). The intra- and interassay coefficients of variation were 12.2% and 14.6%, respectively.

2.4. HPLC separation of progestagens and oestrogens

To obtain information about the polarity and identity of the immunoreactive progestagens and oestrogens in faeces, samples from early and late gestation and from the follicular and the luteal phases of the oestrous cycle were separated using straight phase HPLC. The procedure for separating progestagens was similar to that of Schwarzenberger et al. (1991). Briefly, the extracts were chromatographed on a Lichrosorb Si 60 column using a mixture of chloroform/*n*-hexane (7:3; v:v) with a linear gradient of methanol (0–6%). Elution profiles of ^{3}H-progesterone and ^{3}H-20α-hydroxyprogesterone, added prior to extraction were determined using a liquid scintillation counter, and immunoreactive progestagens were analysed in the EIA.

For the chromatography of oestrogens, ^{3}H-oestrone and ^{3}H-oestradiol-17β were added to the faecal samples before extraction using the same method as that for progestagens, except the mixture of *n*-hexane/chloroform was 1:1 (v:v) and the linear methanol gradient ranged from 0–10%. Immunoreactivity in HPLC-fractions was determined by EIA.

2.5. Data analysis

Data are presented as means ± SEM. Results of all analysed cycles ($n = 10$) were combined for the calculation of the mean concentration of the measured hormones. For each cycle, the day of the measured oestrogen peak was designated Day 0. Because faecal samples of different cycles were not collected on the same days each week, data were combined to obtain two mean values per week. These data were checked for normal distribution by the Kolmogorov–Smirnov-test using SigmaStat® (SPSS, Chicago, IL, USA). Basal oestrogen concentration was calculated as the mean concentration of samples ($n = 67$) collected between Days 10 and 35 and Days −14 and −10 of the ovarian cycle. These periods were designated dioestrus and anoestrus, respectively. Basal concentrations of immunoreactive progestagens (20α-OH-P and 20-oxo-P) were calculated from samples ($n = 58$) collected between Days 28 and 35 and Days −14 and 0 of the ovarian cycle. These periods were designated anoestrus and prooestrus, respectively. Mean peak oestrogen values were calculated from the concentration at Day 0, the mean peak in progestagens was calculated from the highest concentration measured between Day 10 and 21 of the ovarian cycle.

3. Results

3.1. HPLC separation of immunoreactive metabolites

Profiles of immunoreactive progestagen metabolites are illustrated in Fig. 1. After HPLC separation of faecal extracts and subsequent EIA analysis, several immunoreactive compounds cross-reacting with both 20α-OH-P and 20-oxo-P antibodies were found. The relative proportion of metabolites varied considerably depending on the reproductive status of the animal. During the luteal phase (Fig. 1a), several polar metabolites were found. The faecal metabolite profile during early gestation (Fig. 1b) differed from that during late gestation (Fig. 1c), with progestagens cross-reacting with

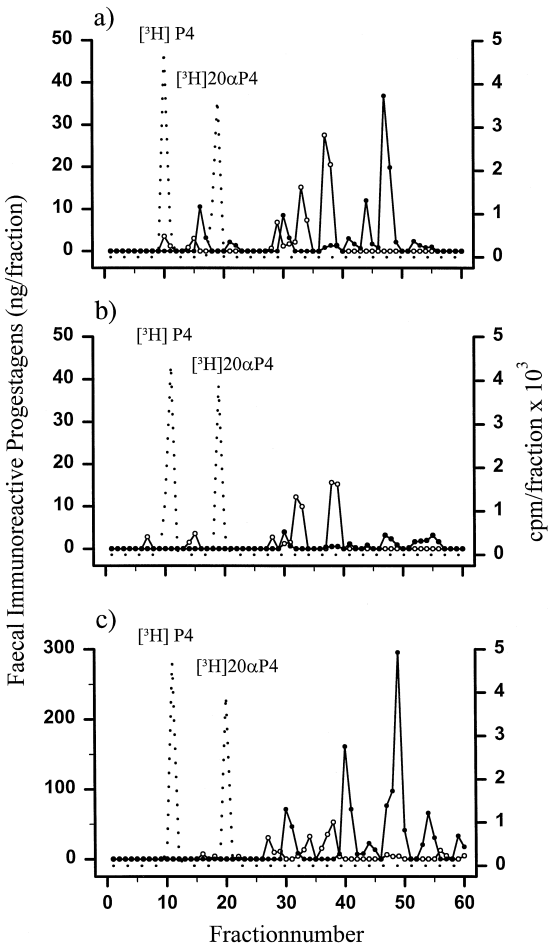

Fig. 1. HPLC separation of faecal immunoreactive progestagens cross-reacting with an antibody directed against 20α-OH-progestagens (●) or against 20-oxo-progestagens (○) during the luteal phase of the oestrous cycle (a) and on Day 45 (b) and Day 122 (c) of pregnancy.

the 20α-OH-P antibody, increasing in number and proportion. Although specific metabolites were not identified, it was evident that none of the immunoreactvity was associated with native progesterone or 20α-hydroxyprogesterone.

In the faecal extract of the follicular phase four apolar peaks all eluting before ^{3}H-20α-hydroxyprogesterone were found (data not shown). Two immunoreactive oestrogen peaks were detected, one coeluting with ^{3}H-oestrone and a second one ~30 times greater proportionally that coeluted with ^{3}H-oestradiol-17β (data not shown).

3.2. Oestrous cycle

Only the three females at the Tierpark Dortmund zoo exhibited regular oestrous cycles. A representative profile of three consecutive cycles is shown in Fig. 2. Fig. 3 depicts the mean profile of faecal oestrogens and 20α-OH-P during 10 complete ovarian cycles. Mean length of the ovarian cycle, defined as the interval between two oestrogen peaks, was 51.4 ± 5.6 days, with a range of 44–63 days. Basal oestrogen concentrations averaged 6.1 ± 0.5 ng/g and increased ~10-fold to a peak of 65.7 ± 9.9 ng/g (range 19.8–99.6) on Day 0. Peak oestrogen concentrations varied considerably within and among individuals. For example, in one animal the peak concentration during the follicular phase in which mating took place was the lowest (19.8 ng/g) of all animals studied, but oestrogen concentrations (99.6 ng/g and 96.6 ng/g) during the second and third follicular phase post-partum were among the highest.

Peak progestagen excretion occurred 16.9 ± 3.7 days (range, 10–21) after the oestrogen maximum. Progestagen concentrations began to increase while the oestrogens were decreasing. In most samples the concentration of 20α-OH-P was greater than that of

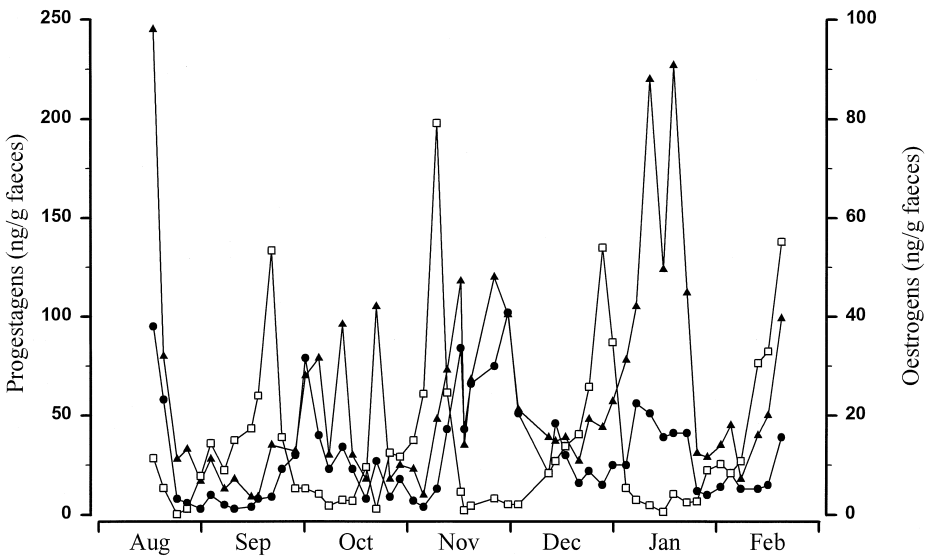

Fig. 2. Individual profiles of faecal immunoreactive 20α-OH-progestagen (▲), 20-oxo-progestagen (●) and oestrogen (□) concentrations depicting normal oestrous cycles over a 6-month period.

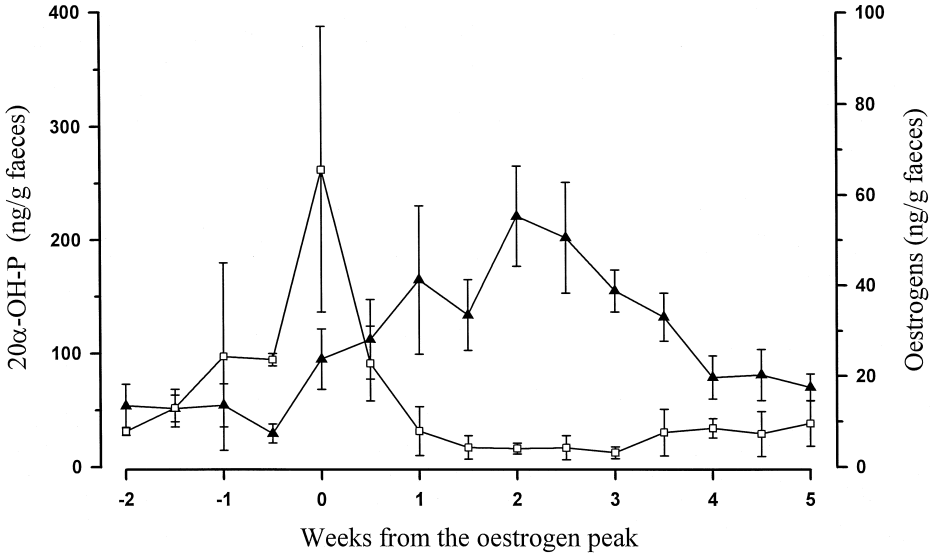

Fig. 3. Mean (±SEM) concentrations of faecal immunoreactive oestrogens (□) and 20α-OH-progestagens (▲) during ovarian cycles in three animals ($n = 10$). Data were aligned to the day of the faecal oestrogen peak.

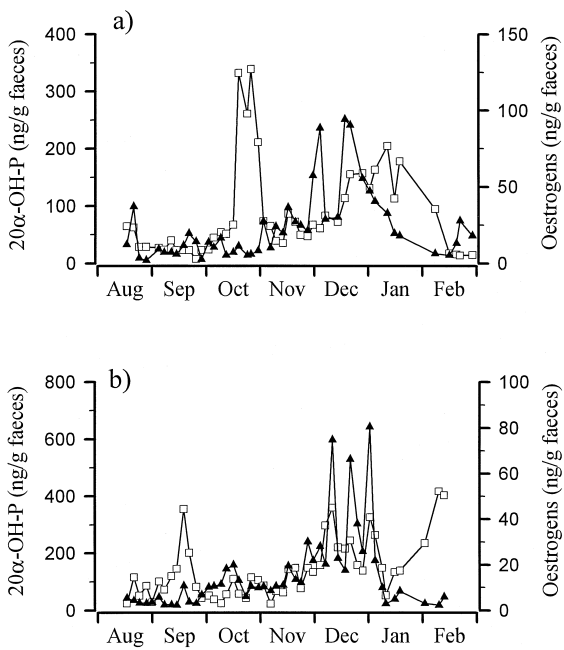

Fig. 4. Concentrations of faecal immunoreactive oestrogens (□) and faecal 20α-OH-progestagens (▲) over a 6-month period in two individuals that did not exhibit clear signs of ovarian cyclicity.

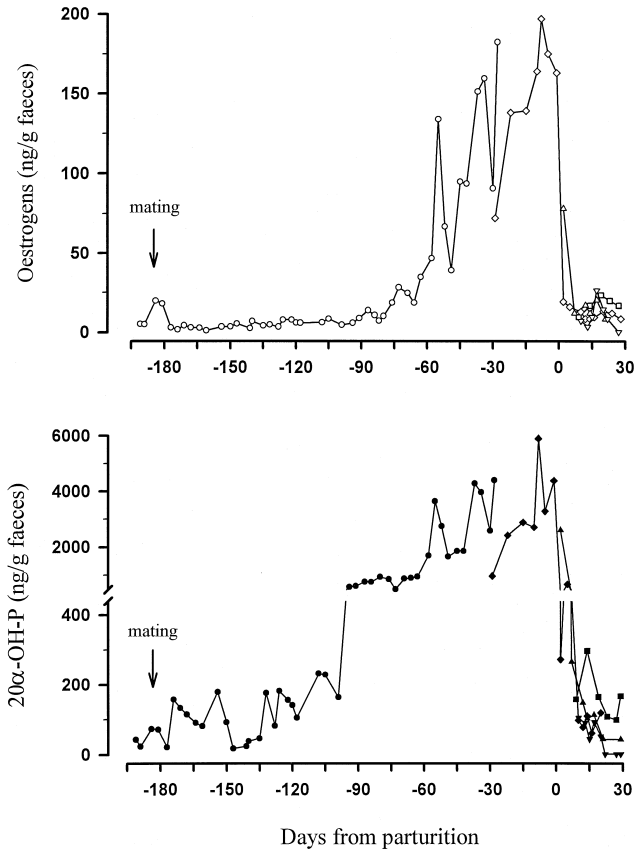

Fig. 5. Concentrations of faecal immunoreactive oestrogens (a) and 20α-OH-progestagens (b) during pregnancy ($n = 2$) and the first month post-partum ($n = 4$) from three females. 0245: one pregnancy (●), and two post partum-periods (■, ▲); 0255: one pregnancy with the following post partum-period (♦) and 0035: one post partum-period (♦).

20-oxo-P. The highest mean concentration of 20α-OH-P during the luteal phase was 244.6 ± 46.7 ng/g (range, 96.3–547.1 ng/g) and 63.5 ± 6.2 ng/g during the inter-luteal period. The respective 20-oxo-P values were 161.2 ± 40.9 ng/g (range 56.3–420.5 ng/g) and 35.8 ± 3.8 ng/g. Because the profile of 20α-OH-P appeared to define corpus luteum activity more clearly, only these profiles were presented in Figs. 3–5.

No regular ovarian cyclicity was observed in the other two females. Faecal oestrogen and progestagen concentrations fluctuated markedly, but randomly (Fig. 4a, b).

3.3. Gestation and the post-partum period

Individual faecal oestrogen and progestagen profiles during parts of two pregnancies (female 0245: Days 0–159, it became pregnant during the first collection period; female 0255) and four post-partum periods are depicted in Fig. 5. Female 0035 was not

pregnant, only two regular ovarian cycles were measured, which were combined with the other data of the ovarian cycle. In early pregnancy, the oestrogen and progestagen patterns were similar to those during the follicular phase, including a decrease in their concentrations about 5 weeks after the oestrogen peak. During gestation, oestrogens increased in the last trimester to concentrations up to 200 ng/g. Concentrations of progestagens increased expeditiously after the second half of gestation, reaching values of >6000 ng/g in the week before delivery. The concentrations of oestrogens and 20α-OH-P decreased after parturition and returned to basal concentrations within 1 week post-partum. Normal ovarian cyclicity resumed at variable times post-partum (at 4, 6 and 10 weeks). In one case, an oestrogen peak was not detected within 15 weeks, and the concentrations of progestagens and oestrogens were lower during that time as compared to the three other post-partum periods.

4. Discussion

This study presents the oestrogen and progestagen profiles during the ovarian cycle and pregnancy of the giant anteater and, hence, expands our knowledge of the reproductive physiology of this species. Metabolites were measured in faecal samples using a simple extraction method before analysis by EIA. Results confirmed that this non-invasive method, as described for many other species (Schwarzenberger et al., 1996, 1997), is applicable for monitoring reproductive status in the giant anteater. Results also indicated that the activity of the ovary and placenta can be assessed sufficiently by collecting faecal samples only twice per week.

The follicular phase lasted 1–2 weeks, followed by a luteal phase of 2–3 weeks, and then a dioestrous/anoestrus lasting about 2–3 weeks. The length of the ovarian cycle varied among the three cycling females, but was more constant within individuals. The ovarian cycle data in this study were comparable to those of the lesser anteater (Hay et al., 1994). Although the average cycle length was about 1 week shorter in the tamandua, the hormonal patterns appeared quite similar for both species.

Our results demonstrated that oestradiol-17β was the major oestrogen metabolite in the giant anteater, and that specific antibodies for oestradiol-17β as well as those for total oestrogens can be used for this analysis (Schwarzenberger et al., 1996). In contrast, because progesterone is metabolised to other compounds before excretion specific antibodies for this steroid are less suitable for analysis of faecal progestagens. Instead, two group-specific antibodies have been found to be more appropriate for assessing faecal progestagens (Schwarzenberger et al., 1996, 1997). During the ovarian cycle and pregnancy, an antibody cross-reacting with 20α-OH-P reflected changes in progestagen concentration better than one cross-reacting with 20-oxo-P. The HPLC results further confirmed these findings because the 20α-OH-P antibody detected more metabolites during late gestation than the 20-oxo-P antibody. The progestagen metabolites in giant anteater faeces were not identified, but Palme et al. (1997) described the elution profiles of several pregnane standards on a comparable straight phase HPLC system which suggested that the metabolites detected in fractions 33–45 appeared to be dihydroxylated 5α- or 5β-pregnanes. Those metabolites eluting later than fraction 45 were more polar than pregnanediols and, therefore, could be pregnanetriols.

The combined measurement of faecal oestrogens and progestagens can provide valuable information about the reproductive status of an animal and help assess irregularities in ovarian cyclicity. It may also be possible to use the oestrogen surge during oestrus to help estimate the best time for breeding. The profile of progestagens alone can be used to monitor ovarian cyclicity satisfactorily if oestrus can be recognised by characteristic behaviour or physical symptoms. In contrast, the giant anteater does not exhibit visible signs of oestrus (Bartmann, 1983), and, therefore, the estimation of oestrogens could facilitate breeding management. The endocrine profiles also were useful for learning more about pregnancy in the giant anteater. The calculated gestation duration was 184 days which was comparable to data published by Bartmann (1983), Moeller (1988) and Poglayen-Neuwall (1990). After mating, the concentrations of steroid metabolites showed the same trend as that during the ovarian cycle. Progestagens declined 5 weeks after mating, a time when the next follicular phase would occur in non-pregnant animals. In the second half of pregnancy progestagens as well as oestrogens increased rapidly, progestagens from the middle of gestation and oestrogens from the third trimester. All analysed hormones decreased rapidly within 1 week after birth. Onset of ovarian activity after parturition showed great individual differences, but no influence of lactation could be recognised, as in all four cases the new-borns were nursed by their mothers for 6–8 months (Bartmann, pers. com.). Additionally, matings occurred during lactation with the interval between subsequent deliveries being $\sim$1 year (Bartmann, pers. com.).

In conclusion, this non-invasive approach to monitoring ovarian function provided valuable information on the reproductive endocrinology of the giant anteater and demonstrated that the measurement of faecal oestrogens and progestagen metabolites was a reliable method for assessing reproductive function in this species. The average cycle length was estimated at $\sim$7 weeks. The appropriate time for mating could be ascertained by increase of faecal oestrogens, and pregnancy could be diagnosed by measuring faecal progestagens that increased markedly above luteal phase concentrations by the second half of gestation.

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