Monitoring ovarian function and pregnancy by evaluating excretion of urinary oestrogen conjugates in semi-free-ranging Przewalski's horses (*Equus przewalskii*)

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Summary. Immunoreactive urinary oestrogen conjugates were assessed in daily urine samples (~5 samples/week) collected from 8 Przewalski's mares maintained under semi-free-ranging pasture conditions. The relative percentage contributions of immunoreactive urinary oestrogens during different reproductive states (oestrus, luteal phase, early, mid- and late gestation) were determined using high-pressure liquid chromatography. In general, conjugated forms of oestrone (oestrone sulphate and oestrone glucuronide) were the major excreted immunoreactive oestrogens in non-pregnant and pregnant Przewalski's mares. Variations in urinary oestrogen conjugates indicated that the onset of oestrous cyclicity coincided with increasing daylengths, and the non-conception oestrous cycle was 24.1 ± 0.7 days ($n = 17$) in duration. Most copulations (29/35, 82.9%) were observed between Day -4 and Day +1 from the preovulatory oestrogen conjugates peak (Day 0). Based on known copulation dates, the mean gestation length was 48.6 ± 0.4 weeks (range 47.3–50.3 weeks). During pregnancy, urinary excretion of oestrogen conjugates increased ~300-fold over levels in non-pregnant mares, reaching peak concentrations by Week +24 (51% of gestation). These results demonstrate that longitudinal reproductive events, including oestrous cyclicity and pregnancy, can be monitored precisely by evaluating urinary oestrogen conjugates in samples from Przewalski's mares maintained under semi-free-ranging conditions.

Keywords: Przewalski's horse; oestrogen; oestrous cycle; pregnancy; urinary steroids

Introduction

Ancestors of the endangered Asian wild horse or Przewalski's horse (*Equus przewalskii*) once roamed throughout Central Asia, China and Europe. The species now is extinct in the wild, and remnant populations exist only in small captive breeding herds. Although evolutionary divergence of the Przewalski's and domestic horse (*Equus caballus*) occurred ~250 000 years ago, the 2 species are capable of interbreeding and producing fertile offspring (Short, 1975). Successful interspecies embryo transfer has also been achieved (Hearn & Summers, 1986). All living Przewalski's horses are descended from 13 founder individuals, the last of which was captured in Mongolia in 1947 (Bowling & Ryder, 1987). Although classified as endangered, the Przewalski's horse reproduces

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successfully in captivity, and the current population now exceeds 900 individuals. Current management strategies focus on broadening the genetic base by minimizing inbreeding and maximizing the genetic contribution from offspring of each of the original 13 founders (Ryder, 1988).

Behavioural studies suggest that Przewalski's mares are seasonally polyoestrous and they exhibit oestrus in early spring after a seasonal anoestrus during the late fall and winter months (Boyd, 1988). Observational studies suggest that mares have a gestation of ~48 weeks (Boyd, 1988).

In the domestic horse, the ovarian hormone oestrone is excreted almost exclusively in the urine (Velle, 1963). Consequently, urinary steroid analysis has been useful for documenting reproductive status in this species (Hillman & Loy, 1975; Palmer & Jousset, 1975). More recently, radioimmunoassay (RIA) of conjugated urinary oestrogens has been used to assess reproductive activity, including diagnosing pregnancy in domestic (Evans *et al.*, 1984; Lasley *et al.*, 1990) and feral (Kirkpatrick *et al.*, 1988) horses and captive zebras (Czekala *et al.*, 1990). Czekala *et al.* (1990) have diagnosed pregnancy successfully in the Przewalski's horse by assessing oestrone sulphate concentrations in urine samples collected by catheterization from anaesthetized mares.

To date, longitudinal endocrine cycles in this species have not been monitored, nor is there specific information on oestrogen metabolism. Details on the latter are a mandatory prerequisite for ensuring the utility of urinary RIA procedures for assessing reproductive function. Our objective was to extend the previous findings of others by using a longitudinal approach. Specifically, the present study was conducted to: (1) validate the RIA procedures for assessing urinary oestrogen conjugates in Przewalski's horses maintained under pasture conditions; (2) use this strategy to characterize seasonal rhythms of ovarian activity and copulatory behaviour in non-pregnant mares; and (3) document excretion patterns of oestrogen conjugates to determine the temporal relationships of copulation to first diagnostic evidence of pregnancy.

Materials and Methods

Animals and sample collections. Urine samples were collected from 3 multiparous and 5 nulliparous mares (2–7 years of age) from May 1987 through November 1988. Mares were maintained with a single stallion in a 12-ha pasture at the National Zoological Park's Conservation and Research Center, Front Royal, Virginia (38° N latitude). Animals were observed (2–4 h/day) at close range from a vehicle, and copulations were recorded. After animals urinated, samples (1–5 ml) were aspirated within 1 min from urine pooled on the ground. Each sample was centrifuged within 3 h (1500 g, 10 min) to remove particulate matter and stored frozen (–20°C) until assayed. Samples were recovered an average of 5 times/week/female throughout the 18-month study period. To compensate for changes in fluid balance, all samples were indexed by creatinine (Cr) measurement (Taussky, 1954; Monfort *et al.*, 1989, 1990), and values were expressed as mass units/mg Cr excreted. To ensure that data were not compromised from urine samples which may have been diluted by water on the ground, samples with Cr concentrations of <0.1 mg/ml were discarded. This criterion eliminated 108 of 1615 collected urine samples (6.7%).

Assay for oestrogen conjugates. Urinary oestrogen conjugates were analysed by a direct RIA procedure described by Shideler *et al.* (1983) using an antiserum (R583; B. L. Lasley, Davis, CA, USA) directed against oestrone-3-glucuronide (1:6000 dilution, 100 µl). With oestrone sulphate as a standard (100%), this antibody cross-reacts with oestrone (200%), oestradiol-17β (100%), equilin (50%), oestrone-3-glucuronide (38%), oestradiol-3-sulphate (21%) and oestradiol-3-glucuronide (6.8%). Cross-reactivity is less than 0.5% for all non-oestrogenic steroids tested (B. L. Lasley, personal communication). Urine samples were diluted 1:100 (25 µl, non-pregnant mares) and 1:10 000 (50 µl, pregnant mares) in Tris buffer (pH 8.4, 0.1 M-Tris, 0.9% NaCl, 1% gelatin) and assayed in duplicate. [³H]Oestrone sulphate (100 µl; 10 000 c.p.m.; sp. act. 60.0 Ci/mmol: Dupont/New England Nuclear, Wilmington, DE, USA) was combined with standards (4.9–1250 pg/tube; in triplicate) and unknowns (in duplicate). After incubation (4°C) for 3 h, 500 µl charcoal–Dextran solution (0.0625% charcoal, 0.00625% Dextran in 0.1 M-phosphate buffer, pH 7.0) were added to separate free from antibody-bound steroid. After a 30-min incubation (4°C), tubes were centrifuged (1500 g, 10 min), decanted into scintillation vials with 5.0 ml Ready Protein (Beckman Instruments, Inc., Fullerton, CA, USA) and radioactivity counted for 5 min.

Parallel displacement curves were obtained by serial dilutions of pooled oestrous, luteal and early pregnancy (~40 days' gestation) urine (1:10 to 1:640) and oestrone sulphate standards. Diluted mid- and late-gestational phase urine (1:400 to 1:100 000) also were parallel to the standard curve. The inter-assay coefficients of variation for 3 separate internal controls were 9.0% ($n = 40$, 20–25% binding), 11.0% ($n = 40$, 50–60% binding) and 26.0% ($n = 40$, 75–85% binding). Inter-assay coefficients of variation were <10%, and assay sensitivity was 4.9 pg/tube. Recovery of

known amounts of oestrone sulphate (range, 4.9–1250 pg/tube) added to a pool of diluted urine (1:100, 25 µl) was $95.1 \pm 8.1\%$ ($y = 1.21 + 1.06x$, $r = 0.99$).

High-pressure liquid chromatography (HPLC). Using pooled urine samples from 2 mares representing different reproductive states (oestrus, luteal phase, early, mid- and late gestation), HPLC was performed to identify the relative contributions of oestrogenic components. The chromatographic system consisted of 2 Rainin Rabbit HP/HPX pumps (Rainin Instruments Co., Woburn, MA, USA) connected to a gradient mixer, a Rheodyne injector (Model 7125, Berkeley, CA, USA) and a 100 µl sample loop fitted to a reverse-phase Microsorb® column (RP C-18, 5 µm diam. particle size). Fractions were collected with a Gilson FC203 fraction collector (Gilson Medical Electronics Inc., Middleton, WI, USA). Chromatographic grade methanol and water were filtered using nylon-66 filters (47 mm diam., 0.2 µm pore size; Rainin Instruments Co.) and degassed before use.

Before HPLC, undiluted urine samples were pre-processed using reversed-phase C18 cartridges (Spice®, Analtech, Newark, DE, USA) according to previously reported methods (Heikkinen *et al.*, 1981). Cartridges were pre-conditioned with 5 ml methanol followed by 5 ml de-ionized water. Urine (0.5 ml) was combined with ~1500 c.p.m. [3 H]oestrone sulphate (to monitor extraction efficiency) in a 3-ml plastic syringe fitted to a C18 cartridge (containing 400 mg sorbent material/cartridge) and loaded into the cartridge. Oestrogens were eluted in 5 ml methanol, evaporated to dryness under nitrogen gas and reconstituted in precisely 0.5 ml methanol. Mean ($\pm$ s.e.m.) percentage recovery of tritiated label was $85.7 \pm 1.5\%$ ($n = 6$). A 0.2 ml portion of the extractant was transferred into a conical vial containing ~6000 c.p.m. each [3 H]oestrone glucuronide, [3 H]oestrone sulphate, [3 H]oestradiol-17 β and [3 H]oestrone added previously (and taken to dryness) to serve as co-chromatographic markers. A 55-µl portion of the extractant (~1600 c.p.m./radiolabelled marker) was loaded on to the HPLC column, and a linear gradient of 20–100% methanol in water within 80 min (1 ml/min flow rate, 1.0 ml fractions) was used to separate oestrogenic components. Separate samples (200 µl) of each fraction were counted directly for recovery and assayed for immunoreactivity using the RIA for conjugated oestrogens described above.

Statistical analysis. Statistics were performed using the System for Elementary Statistical Analysis (SAS Institute Inc., Cary, NC, USA) on a Vax 8800 microcomputer. Weekly mean hormone concentrations represent the mean $\pm$ s.e.m. of daily hormone values pooled by week of collection. Statistical differences between grouped weekly hormone concentrations were determined by one-way analysis of variance, and multiple comparisons were made using Duncan's Multiple Range Test and a Student's *t* test.

Results

The relative percentage contributions of immunoreactive urinary oestrone, oestradiol, oestrone sulphate, oestrone glucuronide and unknown oestrogenic compounds separated by HPLC are depicted in Fig. 1(a, b). Because oestrone and oestradiol co-eluted in the same fractions, these unconjugated oestrogens were expressed as a combined value. Oestrone/oestradiol, oestrone glucuronide and oestrone sulphate were defined as the immunoreactive peaks which co-eluted with the radiolabelled marker. Unconjugated oestrone/oestradiol (46.5%) and conjugated oestrone sulphate (43.3%) predominated in the urine of oestrous mares (Fig. 1b), whereas oestrone glucuronide and unknown immunoreactive oestrogenic components were lesser contributors at 10.0% and <0.2%, respectively. During the luteal phase, conjugated oestrone sulphate comprised 60.9% and oestrone/oestradiol, oestrone glucuronide and unknowns accounted for 20.2%, 18.4% and 0.5% of excreted oestrogen immunoreactivity, respectively. By early pregnancy (~40 days' gestation), immunoreactive oestrone sulphate and oestrone glucuronide were excreted in approximately equal proportions and comprised most of the voided oestrogen (total, 90.5%) compared to the low contribution of oestrone/oestradiol (5.3%) and unknown compounds (4.2%). During mid- and late pregnancy, clearly immunoreactive oestrone sulphate predominated (mid-, 97.9%; late, 92.7%) over all other components. Figure 1(a) illustrates the relative percentage contributions of unconjugated oestrone/oestradiol compared to the combined total of conjugated oestrone sulphate and oestrone glucuronide. In general, as pregnancy progressed, conjugated oestrone increased markedly with a simultaneous decrease in unconjugated oestrogen excretion.

Based on variations in urinary oestrogen conjugates, onset of oestrous cyclicity coincided with increasing daylengths and, among animals, ranged from February to April. Representative longitudinal oestrogen profiles from 3 mares sampled from January through June are depicted in Fig. 2(b–d). Although 4 mares exhibited cyclic ovarian activity, and copulated early in the

Fig. 1. The relative percentage contribution of immunoreactive urinary oestrogens separated by HPLC.

year (February–March), none of these mares conceived during this time. All conceptions ($n = 9$) occurred in the months of April through November.

Daily mean urinary concentrations of oestrogen conjugates ($\pm$ s.e.m.) representing 17 non-conceptive oestrous cycles from 6 mares are presented in Fig. 3. All sample data were aligned on the day of the oestrogen conjugates peak (Day 0). The interval between consecutive peaks provided an estimated oestrous cycle length of 24.1 ± 0.7 days ($n = 17$). Of 35 observed copulations, 29 (83%) occurred from Day -4 to Day $+1$. Beginning on Day -8 , oestrogen conjugate concentrations increased ~ 7 -fold from basal concentrations (0.04 ± 0.02 $\mu\text{g}/\text{mg}$ Cr) to a preovulatory peak of 0.29 ± 0.03 $\mu\text{g}/\text{mg}$ Cr (Day 0, $P < 0.05$). After an abrupt decline ($P < 0.05$) to 0.10 ± 0.02 $\mu\text{g}/\text{mg}$ Cr by Day $+2$, there was a secondary peak to 0.16 ± 0.03 $\mu\text{g}/\text{mg}$ Cr ($P > 0.05$) on Day $+4$ followed by a decline to baseline by Day $+12$ ($P < 0.05$, 0.07 ± 0.01 $\mu\text{g}/\text{mg}$ Cr).

An overall weekly pregnancy composite profile (Fig. 4) was generated from 9 pregnancies (7 mares) aligned to the day of parturition (Day 0): 4 mares were sampled throughout pregnancy, 2 were studied during the last third of pregnancy and 3 were monitored only during the first 10 weeks of gestation. Based on known copulation dates for 6 mares, the mean gestation length was 48.6 ± 0.4 weeks (range, 47.3–50.3 weeks), and the mean time of conception was best approximated by Week -49 (Fig. 4 inset; 0.16 ± 0.01 $\mu\text{g}/\text{mg}$ Cr). During early pregnancy, mean oestrogen

Fig. 2. Longitudinal profiles of urinary oestrogen conjugates from 3 Przewalski's mares (b–d) sampled from January to June during increasing daylengths (a). Asterisks indicate observed copulations.

conjugates values declined slightly ($P > 0.05$) to $0.10 \pm 0.02 \mu\text{g/mg Cr}$ (Week -46 ; Fig. 4, inset), increased significantly to $0.46 \pm 0.05 \mu\text{g/mg Cr}$ by Week -43 (6 weeks after the mid-cycle oestrogen conjugates peak; Fig. 4, inset), rising an additional ~ 200 -fold to peak pregnancy concentration of $101.90 \pm 34.30 \mu\text{g/mg Cr}$ by Week -25 (51% gestation). After the mid-pregnancy peak, weekly concentrations of oestrogen conjugates decreased steadily to $6.90 \pm 1.20 \mu\text{g/mg Cr}$ by Week -1 and

Fig. 3. Daily mean ($\pm$ s.e.m.) urinary oestrogen conjugates representing 17 oestrous cycles from 6 non-pregnant mares. Hormone values were aligned to the day of the urinary oestrogen conjugates (EC) peak.

were not different from those of non-pregnant mares by Week +1 *post partum* (0.09 ± 0.02 $\mu\text{g/mg Cr}$, $P > 0.05$). Urinary concentrations of oestrogen conjugates 1–2 days *post partum* ranged from 0.04 to 0.16 $\mu\text{g/mg Cr}$. Urine sampling frequency decreased during the post-partum interval because of nervous behaviour of the mares. None the less, there was evidence of possibly increased ovarian activity 10–14 days *post partum* in 3 mares based on increased oestrogen conjugates excretion. An example is the mare depicted in Fig. 5 which exhibited 2 non-conception ovarian cycles followed by a conception cycle and pregnancy. An irregular excretory pattern of oestrogen conjugates was observed *post partum* with metabolite concentrations achieving levels measured before conception.

Discussion

This study demonstrates the utility of longitudinal profiles of urinary oestrogen conjugates for studying reproductive seasonality and the endocrine events associated with cyclicity, pregnancy and parturition in the Przewalski's horse. Specifically, these data confirm that Przewalski's mares are seasonally polyoestrous with oestrous cycles commencing in the early spring. The failure of all mares to conceive during February and March suggests that the first seasonal oestrous periods in the Przewalski's horse may be anovulatory, like those in the domestic horse (Ginther, 1974). The finding that conception only occurred from April to November affirmed that this species has a potential breeding season of 7–8 months, which is similar to that of domestic horse (Ginther, 1974; Hughes *et al.*, 1975) and donkey (Henry *et al.*, 1987).

Chromatographic analysis revealed that, in general, conjugated forms of oestrone (oestrone sulphate and oestrone glucuronide) were the major excreted immunoreactive oestrogens in non-pregnant and pregnant Przewalski's mares. Almost half (46.6%) of the excreted oestrogen immunoreactivity in oestrous mare urine was unconjugated oestrone/oestradiol. This contrasts with a

Fig. 4. Weekly mean ($\pm$ s.e.m.) urinary oestrogen conjugates representing 9 pregnancies monitored in 7 mares. Hormone values were aligned to the day of parturition. *Inset:* increase in urinary oestrogen conjugates around the time of conception: asterisk indicates significant increase in value in comparison to concentration in non-pregnant mares.

Fig. 5. Daily urinary excretion of oestrogen conjugates from a single mare sampled from 11 weeks before conception (-400 days) until 6 weeks *post partum* ($+40$ days). Hormone values were aligned to the day of parturition. The vertical axis is presented on a logarithmic scale to facilitate identification of cyclic hormonal patterns. Asterisks denote observed copulations.

report by Lasley *et al.* (1990) which indicated that 95% of the total oestrogen immunoreactivity in the domestic horse was contributed by oestrone sulphate. Unconjugated oestrone/oestradiol also accounted for a substantial proportion of the oestrogen immunoreactivity during the luteal phase ($\sim 20\%$) of the Przewalski's horse, although during pregnancy, oestrone/oestradiol excretion was diminished. During early pregnancy (~ 40 days), oestrone glucuronide was a major contributor ($\sim 43\%$) to total urinary oestrogen immunoreactivity. In contrast, at the same stage of gestation, oestrone glucuronide excretion contributes $< 3\%$ of total immunoreactivity detected in the domestic horse (Evans *et al.*, 1984; Lasley *et al.*, 1990). However, Heap *et al.* (1982) indicated that endometrial tissues of the latter species are capable of converting large quantities of radiolabelled oestrone to oestrone glucuronide (34.0%) and oestrone sulphate (43.6%) by 28–36 days of gestation. Chromatographic separation revealed that 93–98% of excreted oestrogens at mid- and late gestation in the Przewalski's horse are in the form of oestrone sulphate, which further supports our assertion that oestrone is the dominant oestrogen of pregnancy in this species.

The estimated oestrous cycle length of 24.1 ± 0.7 days was shorter than that reported for the donkey (25.4 days: Henry *et al.*, 1987), but longer than that of the domestic horse (21.5 days: Back *et al.*, 1974; 21.7 days: Hillman & Loy, 1975; 20.6 days: Hughes *et al.*, 1975). Mean urinary concentrations of oestrogen conjugates in the Przewalski's horse during the week preceding oestrus (dioestrus, 0.04–0.10 $\mu\text{g}/\text{mg Cr}$) and during oestrus itself (0.10–0.29 $\mu\text{g}/\text{mg Cr}$) were in accord with previous values reported by Czekala *et al.* (1990) for this species. The domestic horse excretes more conjugated oestrogens during dioestrus (0.13–0.15 $\mu\text{g}/\text{mg Cr}$) and oestrus (0.35–0.47 $\mu\text{g}/\text{mg Cr}$) (Evans *et al.*, 1984) than does the Przewalski's horse. Despite quantitative differences, the temporal interrelationships between copulatory behaviour and rising oestrogen excretion levels were similar between the Przewalski's and domestic (Munro *et al.*, 1979) mares.

Urinary concentrations of oestrogen conjugates were elevated markedly by the 6th week of gestation which was the earliest time that a potential pregnancy could be diagnosed accurately. This excretion profile was comparable to that of the domestic horse (Evans *et al.*, 1984; Lasley *et al.*, 1990). In the domestic horse, the sharp increase in urinary oestrogen excretion between 30 and 40 days of gestation appears to reflect increased ovarian activity in response to chorionic gonadotrophin (CG) secreted by the trophoblast (Lasley *et al.*, 1990). Our observations suggest that similar mechanisms may exist in the Przewalski's horse. Because endometrial cups can produce CG in the absence of a fetus (Cole & Goss, 1943), increased urinary excretion of oestrogen conjugates only indicates an attempt to establish pregnancy. Therefore, urinary concentrations of oestrogen conjugates may not reflect actual fetal presence and viability until after endometrial cup production of CG ceases at about 90 days of gestation (Lasley *et al.*, 1990).

Our finding of a gestation length of 48.6 weeks agrees well with estimates reported by Boyd (1988) for this species. The temporal patterns of oestrogen conjugates throughout pregnancy were analogous to the findings of Czekala *et al.* (1990) who examined composite profiles from different mares sampled once or twice during gestation. However, we observed peak urinary concentrations of oestrogen conjugates which exceeded 100 $\mu\text{g}/\text{mg Cr}$, and these concentrations were 25–50% higher than the maximal levels measured by Czekala *et al.* (1990). Peak urinary excretion of oestrogens in the domestic horse occurs at 6–7 months of gestation (Raeside & Liptrap, 1975; Nett *et al.*, 1975), which is about 30 days later than in the Przewalski's mare (5–6 months). However, in animals of both species there is a rapid decline in oestrogen excretion after mid-pregnancy with the decrease becoming more gradual as parturition approaches. The marked increase and subsequent decline in oestrone secretion during pregnancy in the domestic horse parallels fetal gonadal development and regression (Pashen & Allen, 1979; Pashen *et al.*, 1982).

From a conservation and management perspective, our results indicate that longitudinal reproductive events can be monitored precisely by evaluating urinary oestrogen conjugates in samples collected from captive Przewalski's mares maintained under semi-free-ranging conditions. Longitudinal sample collection under field conditions is time-consuming, but provides an effective, non-invasive method for diagnosing pregnancy. Although this approach may not permit tracking

fetal viability until ~25% gestation, we detected no false positives ($n = 9$) in our study. For field diagnosis of pregnancy, we speculate that as few as 3 urine samples collected over a 2-week interval are necessary to predict pregnancy accurately. When stallions are interchanged in an effort to enhance genetic diversity within breeding herds, information on pregnancy status can be used to help prevent infanticide which occurs in this species. We presently perform pregnancy checks before introducing new stallions to the breeding herd, and pregnant mares are removed to a nursery herd for foaling. Finally, longitudinal monitoring of urinary oestrogen metabolites, including prediction of ovulation, could be extremely useful in conjunction with artificial insemination, ovulation induction and embryo recovery. These techniques could be particularly important for ensuring reproduction between valuable but behaviourally incompatible pairs and eliminate the risks inherent in transporting animals between breeding facilities.

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