A comparison of urinary hormone profiles with fecal extract hormone profiles of *Equus ferrus przewalskii*

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Abstract

Przewalski's horse (*Equus ferrus Przewalski)*, is the last remaining cousin of the domestic horse. Due to hunting, habitat loss and severe weather, the wild population declined and was declared extinct in 1969. The current captive population was derived from only fourteen founders, which has resulted in reduced genetic diversity. A sound understanding of the Przewalski's horse's reproductive parameters is necessary in order to utilize artificial reproductive technologies as part of a captive breeding program.

Reproductive hormone levels can be detected in urine, feces, saliva, and blood. In the Przewalski's horse, urine hormone analysis is deemed the only noninvasive, accurate representation of hormone levels. However, urine collection can be problematic and time-consuming, making it difficult to acquire samples from other zoological institutions.

If fecal hormone analyses were found to be an acceptable alternative to urine, more institutions may be willing to participate in studies. This would greatly expand the sample number and yield a more accurate synopsis of the Przewalski's horse estrus cycle.

The objective of this study was to determine whether feces could be used as an alternative to urine for the measure and analysis of the reproductive hormones pregnane (PG) and estrone conjugates (EC). Urine and fecal samples were collected from four Przewalski's horse mares for a period of four weeks. Fecal samples were extracted using a boiling method and extraction efficiency was determined. Both urine and fecal samples were processed and then analyzed for PG and EC using a validated enzyme-linked immunosorbent assay procedure. Fecal samples from three of the mares were also analyzed for estradiol-17β (E2).

Pearson correlation coefficient tests were run on the data to determine the relationship between urine and fecal EC levels (ECU vs. ECF), fecal and urine PG levels (PGF vs. PGU), and urine EC levels and fecal E2 levels (ECU vs. E2F). Statistically significant ($\alpha<0.01$) correlations were found in the PGF vs. PGU analysis for each of the mares (n=4). No statistical significance was found for the remaining tests.

The process of hormone extraction from feces used in this study appears to be adequate for PG analysis. However, this was not found to be an accurate representation of EC and E2 levels. Due to the small sample size, further research is necessary to determine whether adjustment of extraction or ELISA procedures could improve detection of estrogen levels in fecal samples from the Przewalski's horse.

Background and Literature Review

Przewalski's horse (*Equus ferrus przewalskii*) is the last remaining wild cousin of the domestic horse (*Equus caballus*). It originally resided in the steppes of Western Europe and Asia, but the species gradually declined throughout the 1900's due to hunting, loss of habitat to livestock, and severe weather [3] and, as a result, was declared extinct in the wild in 1969.

The entire current Przewalski's horse population in North America can be traced back to fourteen founders that were captured during the 20th century [7]. The small number of founders combined with several years of inadequate breeding management lead to a significant degree of inbreeding in the population. This then produced problems with fertility, decreased fecundity and reduced genetic diversity [3]. The demographics of the North American Przewalski's horse population show that a large portion of the

population is ten years of age or older [8]. In 2003, this structure was deemed unstable by the American Zoo and Aquarium Association's Species Survival Plan Program (SSP).

The SSP invited the Smithsonian National Zoo's Department of Reproductive Sciences to spearhead a study to determine value and effectiveness of assisted reproductive technologies (ART) in improving the reproductive success of the Przewalski's horse population. ARTs have often been used in domestic horses to successfully surmount reduced fertility issues [6] and it is predicted that it will benefit the Przewalski's horses as well [9]. Wildt, D. E. (1992) described the benefits of using ART in dire conservation efforts. ART can facilitate reproduction between behaviorally incompatible, but valuable, animals; allow the exchange of genetic material between geographically separated individuals while limiting the cost and risks of transporting live animals; maximize the use of more valuable genetic material; allow for the retrieval and utilization of gametes postmortem; and provide cryopreserved genetic material from deceased individuals to maintain genetic lines long after its death.

Development of artificial insemination (AI), a form of ART, has been used as an effective method of genetic management in other large mammals, such as the scimitar horned oryx and Eld's deer [4] and it is believed that similar success can be achieved in Przewalski's horses as well.

In order for ART to be successful, a strong, sound understanding of the mare's reproductive biology and estrous cycles (including time of ovulation) is necessary. Although Przewalski's horses likely have a similar estrous cycle to that of the domestic horse, it cannot simply be assumed so, especially since there have been differences found between other breeds of domestic horse [1, 2]. A Previous study showed similarities

between the estrous cycles of the Przewalski's horse and domestic horse, yet only estrogen conjugates were measured in a small number of mares [5].

Reproductive hormone levels can be detected in urine, feces, saliva, and blood. In the Przewalski's horse, urine hormone analysis is deemed the only noninvasive, accurate representation of hormone levels. It was generally believed that an accurate hormone analysis could not be obtained from equid fecal matter due to the high amount of fibrous material and the uneven distribution of substances throughout a single elimination. However, experience shows us that there are many difficulties in collecting viable urine samples from a wild horse, including contamination, natural elements and weather, and general complications and dangers associated with working around large wild animals.

Feces are easier to collect and require less urgency of collection than urine. If fecal hormone analyses were found to be an acceptable alternative to urine, more institutions may be willing to collect samples and participate in studies. This would greatly expand the sample number and yield a more accurate synopsis of the Przewalski's horse estrous cycle.

Objective and Hypothesis

The objective of this study was to determine whether feces could be used as an accurate alternative to urine for the measure and analysis of reproductive hormones in the Przewalski's Horse.

The experiment was designed to compare the levels of pregnane, estradiol-17β, and estrone conjugates between urine and fecal samples collected concurrently over a four week period from mares housed at the Smithsonian Institute's Conservation and Research Center in Front Royal, Virginia (n=4). It is our hypothesis that, if properly collected and

processed, feces can be used to accurately portray the hormone profiles of a Przewalski's Horse.

Materials and Methods

Urine and fecal samples were collected concurrently for four weeks from four Przewalski's horse mares (age 10-25 years) housed at the Smithsonian Institute's Conservation and Research Center in Front Royal, Virginia.

One to five ml urine samples were aspirated from the ground within two minutes of urination from each mare four to seven days of the week between 0700 and 1200 h. Samples were centrifuged at 1500 RPM for 20 minutes to remove particulate. The urine samples were stored at -20°C until processed. Creatinine, a protein released from muscle tissue breakdown and excreted in the urine at a constant rate, was measured in each sample to account for differences in urine concentration. A 1:50 urine:buffer dilution was made for each sample for the creatinine analysis. The standard stock for creatinine was diluted to concentrations of 100, 50, 25, 12.5, and 6.25 µg/ml to determine the standard curve. 50µl per well of standards and samples were added in duplicate to Dynatech Immulon 4 Flat Bottom Plates. Then 50µl each of distilled water, 0.75M NaOH, and 0.4M picric acid were added to each well using a repeater pipet. The plate was gently shaken to mix the substances, incubated at room temperature for 30 minutes, then read at 490nm using a spectrophotometer, determining the concentration of creatinine in each sample as compared to the standard curve.

Fecal samples were obtained by collecting an entire single fecal elimination and thoroughly mixing to create a homogenous distribution of substances. A sample of the blended feces was removed and stored at -20 ºC. Fecal samples were freeze-dried in a

lyophilizer, then crushed and sifted to remove bulk fibrous material. Around 0.2g fecal powder was used for boiling hormone extraction.

The weighed fecal powder samples were added to labeled test tubes and radioactive tritium tracer for a specific hormone was added to each sample to determine recovery following extraction. Five ml of 90% ethanol (EtOH) was added to each test tube and the test tubes were vortexed briefly. All test tubes were boiled in a hot water bath at 96-99º C for 20 minutes and 100% EtOH was added to the samples as needed to prevent them from boiling dry. After boiling, the volume of each of the samples was brought back up to the pre-boil level by adding 100% EtOH. The samples were then centrifuged at 2500 RPM for 20 minutes and the supernatant extract was poured off into a second set of labeled test tubes.

Five ml of 90% EtOH was added back to the test tubes containing the fecal pellet and then vortexed and centrifuged again. The extract from these test tubes was poured off into the second set of test tubes containing the first extract and the fecal pellet was discarded. The extract was dried down under air in a warm water bath and resuspended in 3 ml 100% EtOH, vortexed, sonicated, and dried down again. The extract was then resuspended with 1 ml of 100% methanol (MeOH). A measured portion was transferred to a set of labeled test tubes, used to determine extraction efficiency, and the rest was dried down and stored frozen. The remaining extract was used to make a 1:10 dilution in dilution buffer for use in assays.

Fecal extract in MeOH was added to scintillation vials with scintillation fluid. A Beta Counter was used to determine disintegrations per minute (DPM) of the samples, two blanks for zero values, and two total counts containing 100% tracer. The average

blank value was subtracted from all of the sample values and the average total count to correct for any background radiation. The sample values were then analyzed against the total count value to determine the percent recovery for each sample. Samples with an extraction efficiency of greater than 70% and less than 100% were accepted and all others were re-extracted and tested for efficiency a second time. If the percent recovery did not fall in the acceptable range, then the sample was dismissed from the study. All data was adjusted according to its extraction efficiency.

Random undiluted (neat) samples of both urine and fecal extract were separately pooled for each mare, serially diluted, analyzed, and plotted with the standard curves for estrone conjugates (EC) and pregnane (PG) and estradiol-17β (E2; n=3) to create parallelisms. From the parallelisms, the dilution nearest to 50% binding on the standard curve was determined for each mare and all samples were diluted accordingly.

Samples were analyzed using a validated competitive single antibody enzyme-linked immunosorbent assay (ELISA) procedure [5]. The ELISA procedure is similar for all hormones measured (PG, EC and E2) and for both urine and fecal extract samples. The procedural differences include the type and concentration of hormone antibody and labeled antigen, the assay controls, and incubation times.

The assay began by passively adsorbing a hormone-specific antibody to a Nunc polystyrene microtitre 96 flat well plate. After an incubation period any unadsorbed antibody was washed away and standards (serially diluted from stock), controls and samples were added to the wells. The standards contained a known concentration of antigen and were used to develop a standard curve to determine hormone concentration in the samples. Controls were used to monitor quality control between individual assays.

Hormone-specific enzyme conjugate horseradish peroxidase (HRP) was added to each well. This caused competition between the labeled antigen (HRP) and unlabelled antigen (standards, controls, standards) for binding to the adsorbed antibody receptor sites.

After another period of incubation the unbound antigens were washed away and an azino-bis-3-ethyl benzthiazoline-6-sulfonic acid (ABTS) chromagen and hydrogen peroxide catalyst substrate was added, which reacted with the bound enzyme conjugate to cause color change. The plate was then read by a spectrophotometer plate reader, creating a standard curve and determining the concentration of unlabeled antigen in the samples.

Results and Discussion

Pearson correlation coefficient tests were run on the data to determine the relationship between urine and fecal EC levels (ECU vs. ECF), fecal and urine PG levels (PGF vs. PGU), and urine EC levels and fecal E2 levels (ECU vs. E2F). The results are shown in figure 1.

		Mare 1	Mare 2	Mare 3	Mare 4
PGF/PGU	R	0.94704*	0.75053*	0.71785*	0.69787*
	p-value	<.0001*	0.0013*	0.0057*	0.0026*
ECU/ECF	R	0.48996	0.11717	0.2729	-0.3751
	p-value	0.0753	0.69	0.3452	0.1523
ECU/E2F	R	0.54805	-0.06279	0.16194	n/a
	p-value	0.0425	0.8241	0.5802	n/a

Fig. 1 Pearson Square correlation coefficient (R) and p-value for each test and each mare (n=4). Note: No data available for Mare 4 ECU/E2F test. $\alpha < 0.01$; asterisk indicates statistical significance

Statistically significant ($p<0.01$) correlations were found in the PGF vs. PGU analysis for each of the mares (n=4). No statistical significance was found for the remaining tests. Figures 2 and 3 show urine and fecal PG profiles for Mare 1. This gives a visual comparison of the hormone profile trends.

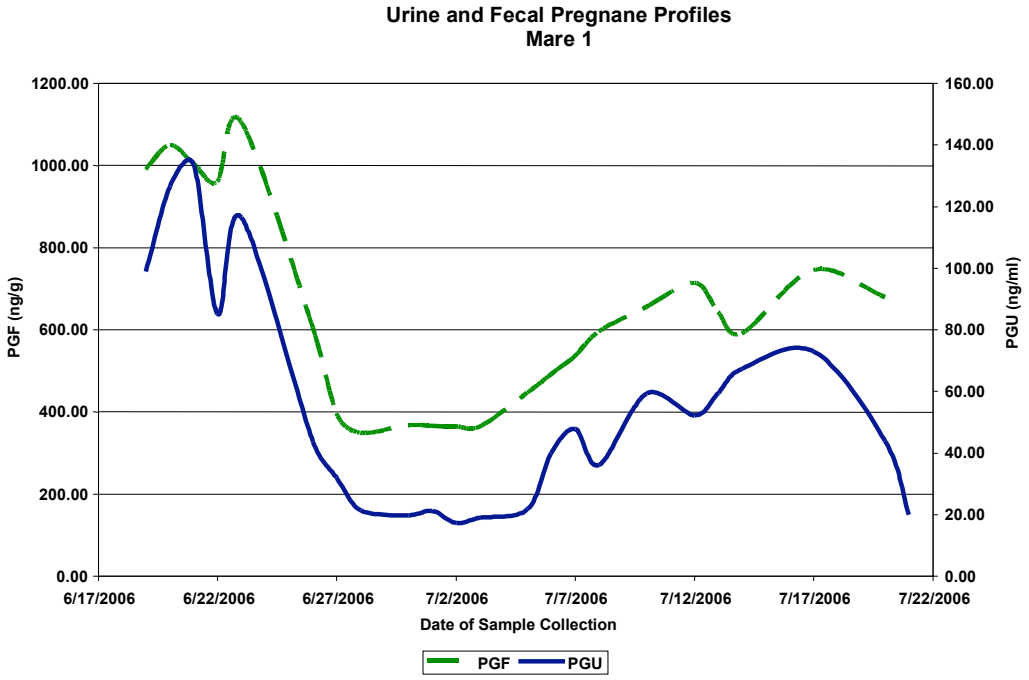

Fig. 2 The profiles of Mare 1 for PGF and PGU are plotted on the same graph to show the relative patterns of hormone fluctuation. PGF is measured in ng/g fecal powder and PGU is measured in ng/mg creatinine.

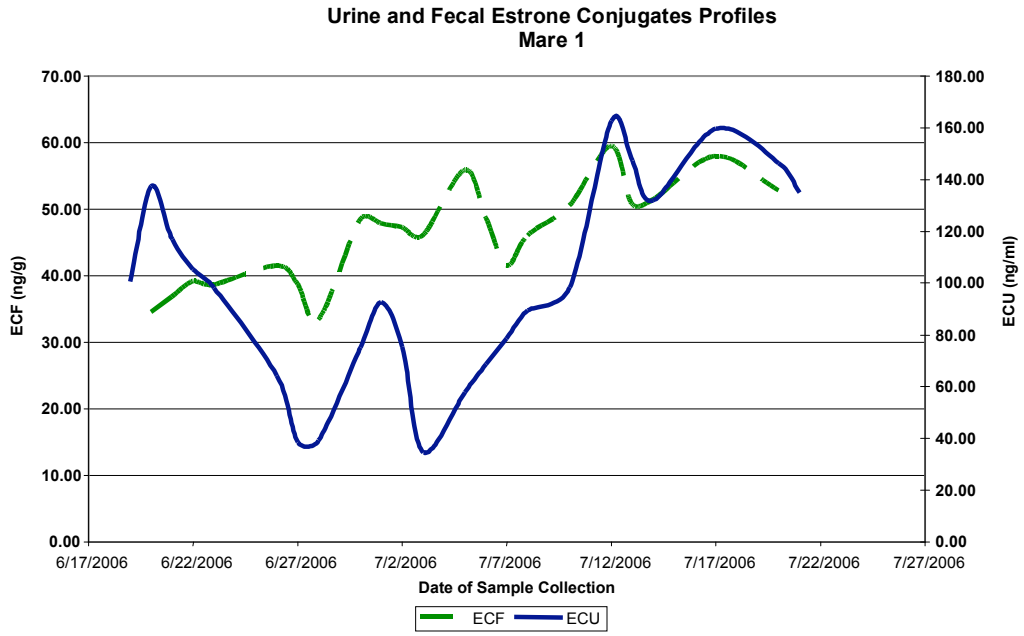

Fig. 3 The profiles of Mare 1 for ECF and ECU are plotted on the same graph to show the relative patterns of hormone fluctuation. ECF is measured in ng/g fecal powder and ECU is measured in ng/mg creatinine.

Figure 2 shows similarity in hormone fluctuation between urine and fecal samples, which is substantiated by the statistically significant correlation coefficients (Fig. 1). Similarly, the relatively erratic ECF profile in figure 3 shows little similarity to the ECU profile, therefore yielding statistically insignificant results (Fig. 1).

The process of hormone extraction from feces used in this study appears to be adequate for PG analysis. However, this was not found to be an accurate representation of EC and E2 levels. There are several possible reasons for the differences observed.

Estrogens may be metabolized and broken down in a different way or at a different rate in the bowel as compared to the urinary tract, which could result in the formation of a different dominant metabolite(s) of estrogen that is/are not recognized by the antibodies used in the EC and E2 assays. A high performance liquid chromatography (HPLC) trial could be run to attempt to identify the dominant metabolite and adjust or create a new assay accordingly.

The fecal hormone extraction procedure used may not be sufficient to fully extract the hormones being measured. Another possibility is that the boiling method may break down or destroy the estrogens such that they are no longer detectable. Another method of extraction such as shaking or vortexing could be used to remove the element of heat.

Further research is necessary to determine the reason for these discrepancies and a larger sample size should be used for further analysis and before making definitive conclusions.

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