PREGNANCY DETERMINATION IN UNCAPTURED FERAL HORSES

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Abstract: The urinary excretion of estrone sulfate (E$_1$S) by 25 free-roaming feral horses (*Equus caballus*) was measured by radioimmunoassay applied to extracts of urine-soaked soil. Twelve of 15 mares having E$_1$S concentrations >1.0 μg/mg creatinine ($\bar{x}$ = 2.64 ± 1.02 [SD]) produced foals. All 10 mares with E$_1$S concentrations <1.0 μg/mg creatinine ($\bar{x}$ = 0.44 ± 0.28) did not foal. Extracting urine from soil and measuring E$_1$S and creatinine can be used to determine pregnancy in free-roaming feral horses without the stress of capture or immobilization.

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The ability to determine pregnancy in free-roaming animals is poorly developed. Birth rates are an inadequate index because they do not provide information about fetal losses, which are vital in understanding nutrition, general health, or stress. To accurately assess pregnancy in wild animals, individuals are usually trapped or immobilized, blood samples taken, and pregnancy determined by measuring estrogen or progestin concentrations or some other specific pregnancy test (Kirkpatrick 1980). These methods, though adequate for domestic animals, frequently are cumbersome, expensive, and disruptive or severely disturbing when applied to wild animals (Seal et al. 1985).

The concentrations of urinary steroids or their conjugated metabolites that change during pregnancy have been measured in many species. Captive animals in which pregnancy has been determined by measuring urinary estrogen or progestin metabolites include the gorilla (*Gorilla gorilla*) (Hopper et al. 1968, Czekala et al. 1983), ruffed lemur (*Lemur variegatus*) (Shideler et al. 1983*a*), vervet monkey (*Cercopithecus aethiops*) (Setchell et al. 1980), lion-tailed macaque (*Macaca silenus*) (Shideler et al. 1985*b*), rhesus monkey (*Macaca mulatta*) (Liskowski et al. 1970, Liskowski and Wolfe 1972), baboon (*Comopithecus hamadryas*) (Hodges et al. 1986), Indian rhinoceros (*Rhinoceros unicornis*) (Kassam and Lasley 1981), and horse (Raeside and Liptrap 1975, Evans et al. 1984, Kasman et al. 1984). Poole et al. (1984) measured testosterone concentrations in elephant (*Loxodonta africana*) urine samples deposited on the ground; urine samples were collected by aspirating the pool of urine minutes after urination occurred. Steroid analysis of urine-soaked soil samples could be an easy, accurate, and inexpensive method to determine pregnancy in free-roaming species with a minimum of disturbance to the population being studied.

Our objective was to develop a test of pregnancy in free-roaming feral horses that is accurate, inexpensive, does not require capture or handling, and does not disturb the population being studied. Specifically, we tested for pregnancy by measuring a urinary estrogen metabolite conjugate, estrone sulfate, in urine-soaked soil.

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METHODS

Urine samples were collected from 25 feral mares on the Pryor Mountain National Wild Horse Range, Montana. Twenty-one of these horses were of known age. In August 1985 feral horses were observed from 100 to 300 m with 8× binoculars. Urination by a mare was identified by her characteristic stance and raised tail. Immediately after urination we located the precise site where urination occurred. In some cases the urine was pooled on hard ground or in shallow depressions in rocks; we collected this urine with a pasteur pipet. In other instances the urine-soaked soil was removed and placed in a 0.25-L styrofoam cup, sealed, and placed on ice. A control sample of dry soil was also taken ap-

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proximately 1 m from the site of urination and treated in the same manner as the urine-soaked soil. In the Pryor Mountains >90% of the mating by feral horses occurs by 30 June (Feist 1971, Feist and McCullough 1975, Kirkpatrick and Turner 1983). Urine samples were collected >35 days after this date because steroid changes are not detectable in pregnant mares until 35 days after fertilization (Evans et al. 1984). We did not select samples contaminated by stallion urine.

The urine-soaked soil samples were taken to Eastern Montana College, Billings, placed in gauze bags, and compressed by hand until the urine was expressed. Where urine could not be removed in this manner, equal volumes of urine-soaked soil and control soil were weighed and a crude estimation of urine volume was made by calculating the difference in weight. One to 8 volumes of distilled water were added to the urine-soaked soil sample. After vigorous stirring, the dilute urine was recovered by compression in gauze bags. Urine samples recovered with pipets were simply centrifuged to remove sediment.

Urine extracts were pipeted into 500 μL micro-centrifuge tubes, centrifuged for 10 minutes in a micro-centrifuge, and filtered through no. 5 filter paper. Samples were stored at −5 C. Urinary E_1S concentrations were quantified in 25-μL urine samples by radioimmunoassay (Shideler et al. 1983*a*). The coefficient of interassay variation was 6–9% (n = 20) at 29–31% bound on the standard curve. The intra-assay coefficient of variation was 3–5%. Sensitivity was <31 pg. Cross-reactivity of the anti-E_1S serum with estrone (E_1) was >99%. However, in equine urine free steroids represent <10% of the total estrogens and conjugated estrone represents >90% of the total excreted estrogen. Because feral horse urine may be more or less concentrated depending upon available water sources, the concentrations of E_1S were normalized to creatinine concentrations. Creatinine concentrations, which have previously been used to estimate glomerular filtration rates in the horse (Cronwall 1985), were determined in 100 μL urine samples (Tausky 1954). Based on the values derived by Evans et al. (1984) for domestic female horses, E_1S concentrations of >1.0 μg/mg creatinine were used to predict pregnancy.

To confirm that the assay was measuring E_1S quantitatively without interference by soil minerals or other unknowns, 3 feral horse urine samples were diluted 1:2, 1:4, and 1:8; assayed, and compared to the standard curve for parallelism. Three experiments were also performed to assess the quantitative recovery of creatinine and E_1S by the extraction procedures. To assess creatinine recovery, 500-mL samples of water containing 1.0, 2.0, 3.0, 4.0, and 5.0 mg/mL creatinine were poured onto soil of the type found on the Pryor Mountain range, extracted, and measured for creatinine as described above. In a second experiment, 500 mL of distilled water were poured onto 5 randomly chosen sites and the water was recovered and measured for creatinine as described above. Finally, 1×10^6 counts/minute of tritium-labeled E_1S was added to 5 mL of distilled water or diestrus domestic mare urine, slurried with 5 separate soil samples, and incubated for 1 week. The water or urine was recovered as described above and 50 μL of supernatant was counted to determine percent recovery. To confirm that the source of the label was E_1S, a 25-μL aliquot of the recovered supernatant was incubated with 100 μL of anti-E_1S serum (1:5,000 dilution) for 1 hour at 15 C. The free label was separated from bound ligand with 300 μL of charcoal–dextran (30 min at 15 C), centrifuged at 2,500 × g for 10 minutes, and counted for 5 minutes. Counts/minute of the bound ligand was compared to counts/minute of ligand added.

During spring and summer 1986 the feral females used in this study were relocated and identified by band affiliation and unique markings. The females were observed for the presence of foals and/or yearlings, and the foaling rate was compared to the predicted pregnancy rate. Differences between means were tested for significance with a t-test.

RESULTS

Urine volumes recovered ranged from 0.3 to 5.0 mL. The E_1S levels when indexed by creatinine ranged from 0.084 to 5.166 μg/mg creatinine. The dose response curve resulting from serially diluted samples (Fig. 1) demonstrates that this assay produces quantitative changes. Among the 5 control soil samples, recovery of creatinine was 91.3 ± 4.2%(SD). When expected creatinine values were compared to values measured, the coefficient of correlation was r = −0.87. Distilled water blanks recovered from soil gave a mean value of 0.014 ± 0.007 mg/mL. Recovery of confirmed tritium-labeled E_1S was 95.5 ± 2.4% with no differences ($P > 0.05$) between ligand recovered from water or urine.

Fifteen females had E_1S concentrations of >1.0 μg/mg creatinine and 10 females had concentrations <1.0 μg/mg creatinine. Twelve of the 15 females with E_1S concentrations >1.0 μg/mg creatinine had foals at their side in 1986. None of the 10 females with E_1S concentrations of less than 1.0 μg/mg creatinine had foals in 1986.

The mean E_1S concentration for females with foals ($n = 12$) was 2.64 ± 1.02 μg/mg creatinine and differed ($P < 0.01$) from the mean for females without foals (0.64 ± 0.45 μg/mg creatinine, $n = 13$). The mean age of known age females without foals ($n = 11$) (7.4 ± 3.8 yr) was not different ($P > 0.40$) than that of known age females with foals (9.33 ± 5.46 yr, $n = 10$). Five of the 12 females with foals also had yearlings at their sides, and five of the 13 without foals had yearlings.

The 3 females with E_1S concentrations >1.0 μg/mg creatinine that did not foal had lower E_1S concentrations ($P < 0.05$) than the 12 with foals (1.31 ± 0.22 vs. 2.64 ± 1.02 μg/mg creatinine, respectively). However, the E_1S values for these 3 females were not the lowest 3 values recorded for the 15 presumed pregnant mares. All 3 of these females without foals had yearlings at their sides.

DISCUSSION AND MANAGEMENT IMPLICATIONS

The concentrations of urinary E_1S produced by pregnant and nonpregnant feral female horses and derived from urine-soaked soil are consistent with values for domestic female horses (Kasman et al. 1983, Evans et al. 1984). The favorable comparison with values found in domestic female horses, the dose-response dynamics of 0.5–0.0625 μL of soil-extracted urine, and the similarity of slope of these samples to the standard curve suggest that the ligand measures only E_1S. Because feral animals may exhibit great differences in water intake and output, creatinine provides an essential index against which to evaluate E_1S and indicates that adequate urine was present. The high recovery of creatinine in control samples and the absence of measurable creatinine in water blanks supports the validity of this index.

Because most steroids are stable, few special procedures or time constraints were required during sampling. Collection of the entire urine sample is unnecessary as only about 150 μL of urine is required for the requisite assays.

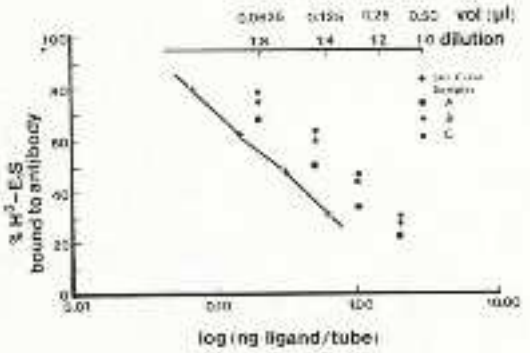

Fig. 1. Standard curve for estrone sulfate (E_1S) assay and dilutions of 3 randomly selected urine samples from feral horses in Montana, 1985.

Fetal losses in feral and wild animals are expected and influenced by many physiological and environmental forces. The 3 females diagnosed as pregnant that had E_1S concentrations >1.0 μg/mg creatinine, but did not foal, all had yearlings at their sides during the spring of 1986. The assumed pregnancy rate was 15 of 25 (60%) and the foaling rate was 12 of 25 (48%). This pregnancy rate of 60% compares favorably with the pregnancy rate of 67.7% for Challis, Idaho, mares, determined by plasma hormone analyses (Seal and Plotka, 1983). The foaling rate of 48% is similar to the 43.2% rate reported by Feist (1971) for Pryor Mountain mares.

Although pregnancy diagnosis in uncaptured large animals is a powerful tool for understanding reproductive biology, our technique has some shortcomings. First, it requires a great deal of time observing animals to collect urine samples. Second, this technique will only measure pregnancy and embryonic loss >35 days after fertilization in horses; thus, very early embryonic loss cannot be determined. Finally, preliminary experiments indicate that certain soil types interfere with assay procedures. Soil types like those found in the Pryor Mountains contain large quantities of bentonite (sodium silicoaluminate) which yield a clear urine. However, dark volcanic soils found on the Challis horse range stained the urine a dark color that could not be removed and made analysis of creatinine by colorimetric test impossible. This problem can probably be overcome by rapid separation of urine from soil or by a noncolorimetric test for creatinine. Despite these problems, the accuracy of this form of atraumatic pregnancy testing is encouraging, particularly as it can be used with-

out subjecting animals to stressful, expensive, and often dangerous forms of restraint.

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