

The diverse history of the feral horse has led to overabundance within the United States, causing significant ecological and economic impacts. Due to a lack of natural predators, many feral horse herds have the capacity for mean annual increases of 20% (National Research Council 2013), resulting in ecosystem strain through depletion of necessary resources and competition with native wildlife (Berger 1985; Coates and Schemnitz 1994).

While management options for feral horses vary, a promising method is through the use of fertility control via the administration of immunocontraceptive drugs to the mares of the populations. As vaccine efficacy exists relative both to frequency of application and drug of use, it is important to determine pregnancy status of the treated mares throughout the duration of perceived efficacy. Although it is possible to determine pregnancy in the mare as early as 35-42 days through blood hormone analysis (Brinsko et al 2011), it is both impractical and dangerous to attempt to do so in a feral mare without restraint. Another means of accurate pregnancy diagnosis in feral mares is therefore necessary, and the development of a reliable, non-invasive technique for monitoring reproductive hormones in the mare is of interest.

Monitoring of steroid metabolites was originally developed in zoos to obtain biological samples without incurring capture and restraint stress. In many species, the feto-placental unit produces large quantities of estrogen, with marked increases at species specific points of gestation (Lasley et al 1991). Therefore, pregnancy has been accurately diagnosed measuring total fecal estrogens in red buffalo (*Syncerus caffer nanus*), yak (*Bos mutus*), Nubian ibex (*Capra ibex nubiana*) and hippo (*Hippopotamus amphibius*) (Lasley et al 1991). Additionally, pregnancy status in the pigtailed macaque (*M. nemestrina*) and Grevy's zebra (*Equus grevyi*) has been successfully determined through measurement of fecal estradiol (Wasser et al 1988; Asa et al 2001).

Since the initiation of measuring steroid metabolites, research has been completed in both domestic and feral horses regarding determination of pregnancy. In the pregnant mare, the main estrogens found in increasing concentrations are estrone, estrone sulfate and estradiol 17 β (Bamberg et al 1984), all of which are found in the feces in measurable amounts. Previous studies have measured both estrone sulfate and total fecal estrogens relative to pregnancy status in feral mares, with definitive determination of pregnancy at least 150 days after breeding (Henderson et al 1999 and Linklater et al 2000) for estrone sulfate, and between 120-180 days post breeding (Kirkpatrick et al 1989) regarding total fecal estrogens.

Prior to the current study, there has yet to be an equine study utilizing fecal estradiol to measure pregnancy status of the mare, although there has been one completed using captive Grevy's Zebra (*Equus grevyi*) mares (Asa et al 2001). Although the gestational length of 390 to 406 days is longer than that of horses, the pattern of fecal estradiol during pregnancy was similar to that of reported for serum estradiol in domestic horses (Terqui and Palmer 1979; Henderson et al 1998), with highest levels occurring mid gestation then declining prior to parturition (Asa et al 2001). This trend for estradiol levels was also seen in a study measuring plasma estradiol in domestic horses, with estradiol concentrations significantly increasing at 150 days post insemination, peaking at 240 days, then gradually decreasing until roughly 30 days prior to parturition, where a precipitous decrease occurred (Nett et al 1973).

Relative to the equid fecal estradiol and equine plasma estradiol studies, this two-part study was designed to examine the reliability of measuring fecal estradiol for use in assessment of pregnancy status in the mare. The first part of this research examined changes in domestic mare fecal estradiol concentrations, using serum estradiol as a comparison tool. The objectives of this study were: 1) to determine a reliable cut-off concentration that clearly delineated between pregnant and non-pregnant mares; specific to both feces and serum and 2) determine the day of gestation in which pregnant mare fecal and serum estradiol concentrations surpassed non-pregnant concentrations. The second part of this study was designed to compare fecal and serum estradiol cut-offs to pregnancy determined in domestic mares to those of fecal and serum samples from feral

mares. The objectives of this study were 1) to correlate findings of measured fecal and serum estradiol concentrations compared to ultrasound results, and 2) to determine the accuracy of pregnancy status from single fecal samples from feral mares.

Study 1: Measurement of Fecal and Serum Estradiol in the Domestic Mare

Objectives

- 1) to determine a reliable cut-off concentration that clearly delineated between pregnant and non-pregnant mares; specific to both feces and serum.
- 2) determine the day of gestation in which pregnant mare fecal and serum estradiol concentrations surpassed non-pregnant concentrations.

Materials and Methods

Collection and Preparation of Feces and Blood

Daily blood and fecal samples were concurrently collected from eight non-pregnant cycling mares, ranging in age from 9 to 11 years. Seven mares were grade, while the eighth was a paint. While mares were not ultra-sounded to determine day of cycle at sample initiation, the average reported length of an equine estrous cycle is 21-22 days (Brinsko et al 2011), so samples were taken from mares for a total of 23 days, with the exception of mare 443, who had samples taken for 26 days. Feces and serum estradiol concentrations were examined separately, and samples aligned such that the highest concentrations were the first in the sample list.

Blood and feces were also collected concurrently on a weekly basis from 8 pregnant domestic mares with known embryo transfer dates of 7-day embryos. Mares were all grade, and at the time of sampling, ranged in age from 6-16 years. Although blood and feces were taken weekly, the mares were asynchronous in their gestational timing, and initiation of sampling was earlier in gestation in some mares than others, dependent on availability of mare for full gestational sampling.

For both groups (non-pregnant and pregnant), fecal samples were obtained via fecal grabs while mares were restrained in standing stocks. Sample sleeves were inverted around sample, and then labeled with mare identification and corresponding date. Directly following fecal collection, jugular blood was drawn using 18-gauge 1.5 inch needles, filling 2 red top vacutainer tubes for each mare at each bleed. Immediately following collection, labeled fecal samples were placed on ice, with tube racks on top, to be kept cool for transport. Fecal samples were processed within 4 hours of collection, while blood samples were left at room temperature overnight. After sitting overnight at room temperature, each blood sample was centrifuged for 30 minutes at 2400xg, and serum placed into 2.0 mL cryogenic vials for storage at -20°C until extraction.

Extraction

Although extraction procedures differ for feces and serum, both methods utilized quality controls (QCs), which were extracted alongside samples. For this study, the QCs were made from hypophysectomized sheep serum, with varying concentrations of estradiol added (Nett 2005). The concentration of the low QC was 15 pg/mL of estradiol, medium 60 pg/mL, and high 240 pg/mL. Additionally, there was a solvent QC, to be extracted with just diethyl ether (Nett 2005). All QC's were extracted at a volume of 250 µL.

Extraction of feces contained one pre-extraction step, and two extraction steps (Nett 2005). Prior to extraction, each raw chilled fecal sample was placed into a 94x16 petri dish in a thin layer that completely covered the bottom half of the dish. Any extra raw fecal matter was placed into a 50 mL conical vial, labeled with mare information and date, and then frozen at -20°C. Each petri dish of feces had a Kim wipe taped over the top for ventilation during lyophilization. The dishes were then frozen at -20°C for 30 minutes prior to placement into a lyophilizer for 72 hours, until dried completely. Each sample was then hand ground to a fine sand-like consistency and placed into a labeled 50 mL conical vial. Ten milligrams of feces for each sample was placed into 16x150 mm glass tubes and rehydrated with 1 mL of double-deionized water (DDH₂O). Resulting sample slurries were agitated for 1-1.5 hours, followed by centrifugation at 2400xg for 15 minutes. Five hundred microliters of supernatant was removed from each sample for use in the first extraction.

Fecal extractions one and two contained washing steps; for each 500 μ L equine sample aliquot in a 16x150 mm tube, two 16x150 mm tubes were labeled with the same sample number, each containing 500 μ L of DDH₂O for washing. Each 250 μ L QC aliquot also had the same number of tubes prepared. For the second extraction, 5 mL of diethyl ether was added to each 500 μ L equine aliquot or 250 μ L QC, and then vortexed for 5 minutes. Samples were snap-frozen in a methanol-dry ice bath, and the organic phases poured into the first of the corresponding clean 16x150 mm tubes containing 500 μ L DDH₂O for washing. Tubes were then vortexed again for 2 minutes, snap-frozen in a methanol-dry ice bath, and the washed organic phases poured into clean 12x75 mm glass tubes, placed into a heating block, and evaporated under nitrogen (Nett 2005). The second extraction was completed by adding 5 mL of diethyl ether to the original aliquots, and the protocol run again, using the second set of washing tubes during the wash phase (Nett 2005). All 495 domestic mare fecal samples were processed in this manner.

Following the second extraction, the 12x75 mm tubes containing samples and QC's were reconstituted with 0.1% PBS-gel at the volume they were extracted at: 500 μ L for the mare samples, and 250 μ L for the QC's (Nett 2005). They were then vortexed for 2 minutes, incubated at 4°C overnight, vortexed again for 2 minutes and then frozen at -20°C until assay.

Serum required two extractions. From each serum sample, a 500 μ L aliquot was placed into a labeled 16x150 mm glass tube, which had a corresponding labeled 12x75 mm labeled glass tube. As with fecal extractions, there were also labeled 16x150 mm and 12x75 mm labeled glass tubes for the QCs to be extracted alongside the equine samples. Five mL of diethyl ether was added to all tubes, which were then vortexed for 2 minutes, and allowed to stand for 5 minutes (Nett 2005). All tubes were then snap-frozen in a methanol-dry ice bath, then organic phases poured into the 12x75 mm tubes, which were placed into a heating block, and evaporated under nitrogen. For the second extraction, the steps from the addition of 5 mL diethyl ether on were replicated, and the same 12x75 mm tubes dried down. All tubes were then reconstituted with 0.1% PBS-gel at the same extraction volume used, 500 μ L for equine samples, and 250 μ L for QCs. All tubes were then vortexed for two minutes, incubated overnight at 4°C, vortexed again for 2 minutes and then frozen at -20°C until assayed (Nett 2005). All 500 domestic mare serum samples were extracted using this method.

Assay

An RIA specific for estradiol 17 β was utilized for this research, and both primary and secondary antibodies used were created prior to the study. The primary antibody was produced in rabbits from estradiol conjugated to bovine serum albumin (E26BSA), while the secondary antibody was produced in a goat.

All fecal, serum, and QC samples were assayed in duplicate, while 6 standards were assayed in triplicate (Nett 2005) and ranged in concentration from 40 pg to 409.6 fg. The amount of equine sample added to each tube was dependent on status of pregnancy, as well as sample type: feces or serum. Extracted fecal samples from pregnant mares were split at gestational day 100; prior to day 100, 100 μ L of extracted sample was added to each tube, after day 100, 10 μ L of extracted sample was used. Extracted serum samples collected from pregnant mares were divided at gestation day 130; prior to day 130, 200 μ L of sample was added to each tube, after day 130, 50 μ L was added. For cycling mares, 200 μ L of sample/tube was added, for both feces and serum. Eighty microliters of each extracted QC was used for QC tubes.

Tubes were numbered prior to start of RIA, with tubes 1-3 the total count tubes (TC- radioactive ligand), 4-6 Non-specific Binding (NSB) tubes, 7-14 QC's, 15-17 Buffer Control (BC-0.1%PBS-gel) tubes, and 18-23 Standards (Nett 2005). Two additional sets of BC and Standard tubes were throughout the protocol, but tube number was specific to individual RIA. Total volume for each tube of sample/QC or standard, plus buffer (PBS-gel 0.1%) was 500 μ L (Nett 2005). Five hundred microliters of buffer was added to NSB tubes and BC tubes. One hundred microliters of iodinated estradiol 17 β -6-TME was added to all tubes in the assay, followed by 200 μ L of 1:400 Normal Rabbit Serum (NRS) to the NSB tubes (Nett 2005). Two hundred microliters of diluted primary antibody (1:400,000 in 1:400 NRS) was added to all tubes except the TC and NSB tubes. All tubes in the assay were vortexed for approximately 5 seconds, then incubated at 4°C for 24 hours (Nett 2005). After 24 hours of incubation, the secondary antibody, 200 μ L of diluted secondary antibody (1:00 goat anti rabbit (GAR) in PBS-

EDTA), was added to all tubes except TC tubes, vortexed, and returned to the 4°C for additional incubation of 72 hours. Seventy-two hours after adding GAR, 3 mL of cold phosphate buffered saline was added to all tubes except TC. Excluding TC tubes, all others were centrifuged for 30 minutes at 2400xg, and resulting supernatant decanted into waste containers. All tubes, including TC, were then counted on a gamma counter with a counting efficiency of 85% (Nett 2005).

The RIA used had 100% cross reactivity with estradiol 17 β , 12% with estrone, 7% with estradiol 17 α , 9% to estriol, and 1% with both testosterone and progesterone (Niswender et al 1969). The sensitivity of the RIA was found to be an average of 228 fg/tube with the 50% dose of the theoretical curve at an average of 6 pg/tube of equine estradiol.

Results

Cycling Mares

The concentration of estradiol 17 β in 185 fecal samples obtained from 8 cycling mares throughout one estrous cycle ranged from 0.2 – 9 pg/mg of feces, with an overall mean of 1.3 ± 0.1 (SEM) pg/mg. The cycling mare values were utilized to create a cut-off concentration (COC) delineating between non-pregnant and pregnant mares, which was calculated by taking the highest fecal estradiol concentration of the cycle for each mare and averaging them, resulting in a concentration of 5pg/mg of feces. The value 2 standard deviations (SD) above this mean was calculated to be 10 pg/mg of feces. When computing the values that would delineate between non-pregnant or pregnant mares (i.e. the cut-offs), calculations were made relative to methods used in the literature. Referenced studies (Bamberg et al 1984; Henderson et al 1998 and 1999; Linklater et al 2000), utilized the overall mean from non-pregnant mares + 3 standard deviations (SD). Instead of using the overall mean of non-pregnant mares in this study, the highest concentration from each non-pregnant mare's cycle was averaged, and 2 SD's added. Of the 185 fecal samples collected from cycling mares, none of them were above the calculated COC, which is depicted in Figure 1.

Serum concentration of estradiol 17 β in 186 samples obtained from the 8 cycling mares throughout one estrous cycle ranged from 0.6 - 43 pg/mL of serum, with an overall mean of 10 ± 0.6 (SEM) pg/mL. The COC for serum was created using the same methods as for feces; the highest serum concentration for each mare was obtained, and all values averaged, resulting in 27 pg/mL serum. The COC 2 SD of this average was calculated to be 46 pg/mL of serum, which eclipsed all individual sample concentrations for the cycling mares, as shown in Figure 2.

Gestational Mares

The average gestational length for mares in this study was 348 days. The range in concentrations of fecal estradiol 17 β in eight gestational mares was 1 - 134 pg/mg of feces. Concentrations increased as pregnancy progressed, surpassing the calculated COC of 10 pg/mg feces at an average of 105 days of gestation (Cut Off Day – COD), before decreasing below the COC directly before parturition, as indicated in both Figure 3A and 3B.

Serum estradiol 17 β concentrations ranged from 1.7 – 447 pg/mL, with concentrations exceeding the calculated COC of 46 pg/mL serum at an average of 128 days (Figure 4A). Of the 317 serum samples, 65 were collected before the COD of 128 days, with 5 samples surpassing the COC of 45.87 pg/mL serum. Of the remaining 252 serum samples collected after the COD, 13 of them failed to surpass the COC. Figure 4B illustrates the serum estradiol concentrations from the 8 gestational mares 30 days pre-partum.

Preliminary Considerations for Study One

The fecal and serum results of this study indicate that measurement of estradiol 17 β is a reliable method for pregnancy determination in the domestic mare. Of the collected fecal samples that exceeded the COD of 105 days of gestation, 96.6% of them remained above the COC of 10 pg/mg for the entirety of gestation. Of the serum samples collected after COD of 128 of gestation, 94.8% of them returned values above the COC of 46 pg/mL serum throughout gestation.

Regarding the fecal estradiol portion of this study, the earliest calculated day of gestation that returned estradiol 17 β concentrations over those of cycling mare values was 105 days of gestation. This COD is roughly 2

weeks – 2.5 months sooner than studies measuring total unconjugated fecal estrogens (Bamberg et al 1984; Kirkpatrick et al 1989), and approximately 1.5 months earlier than those measuring fecal estrone sulfate concentrations (Henderson et al 1998 and 1999; Linklater et al 2000). Dependent on feral population, earlier knowledge of pregnancy status within the herd could be useful in terms of management projections for the following year.

Figures for Study 1

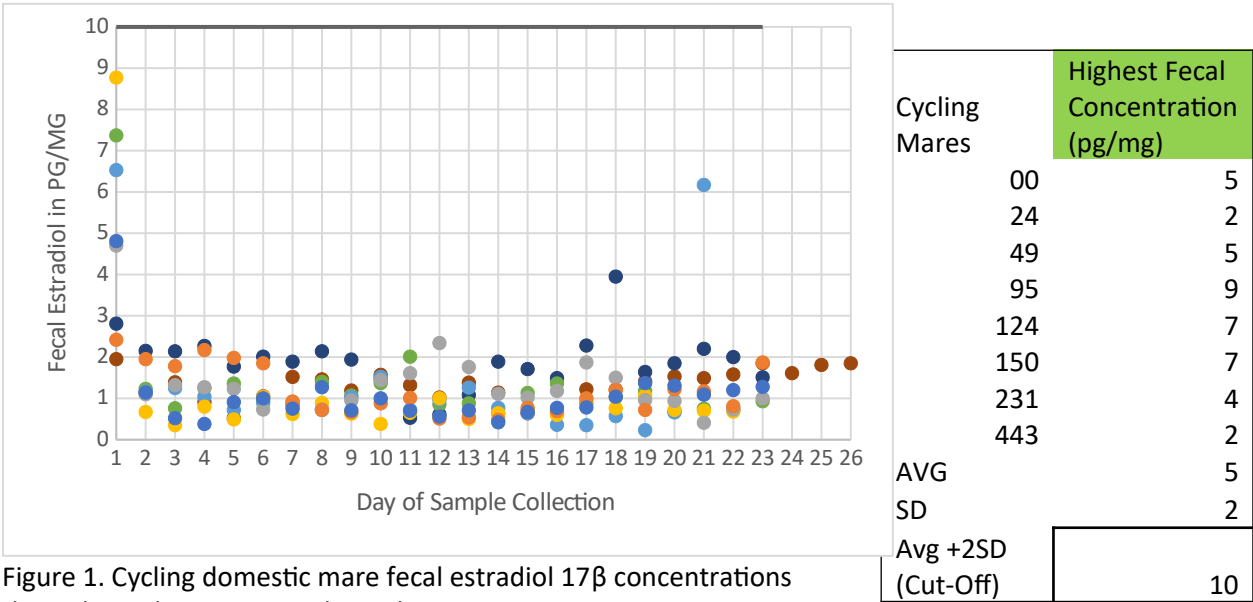


Figure 1. Cycling domestic mare fecal estradiol 17β concentrations throughout the estrous cycle, with included cut-off calculation of 10 pg/mg feces. This value is the mean of the highest fecal estradiol concentration of each mare + 2 standard deviations.

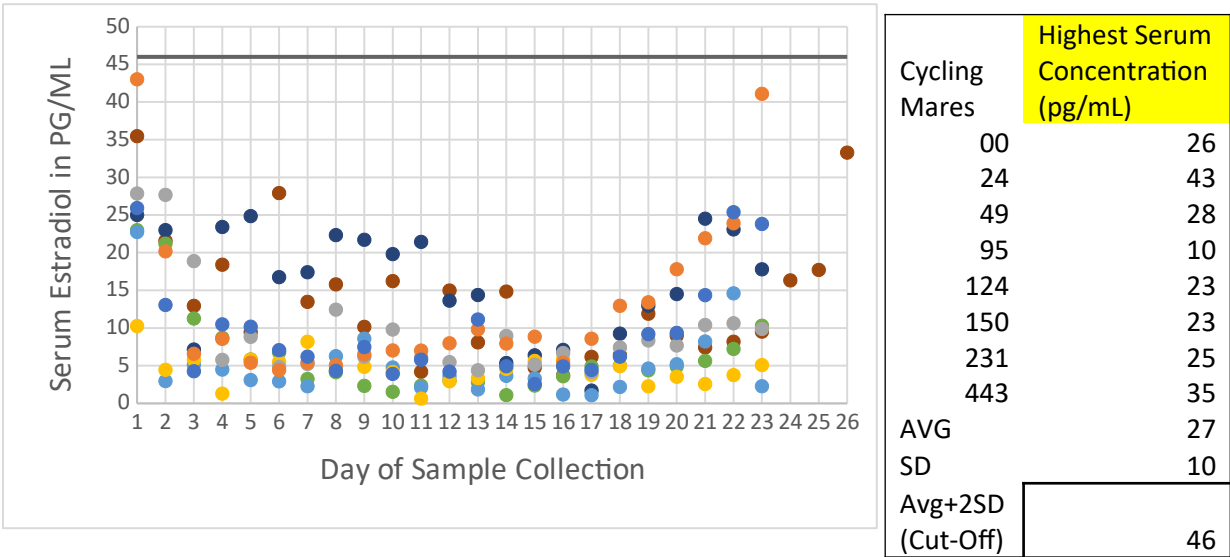


Figure 2. Cycling domestic mare serum estradiol 17β concentrations throughout estrous cycle, with calculations for cut-off value of 46 pg/ml of serum included. This value is the mean of the highest serum estradiol concentration of each mare + 2 standard deviations.

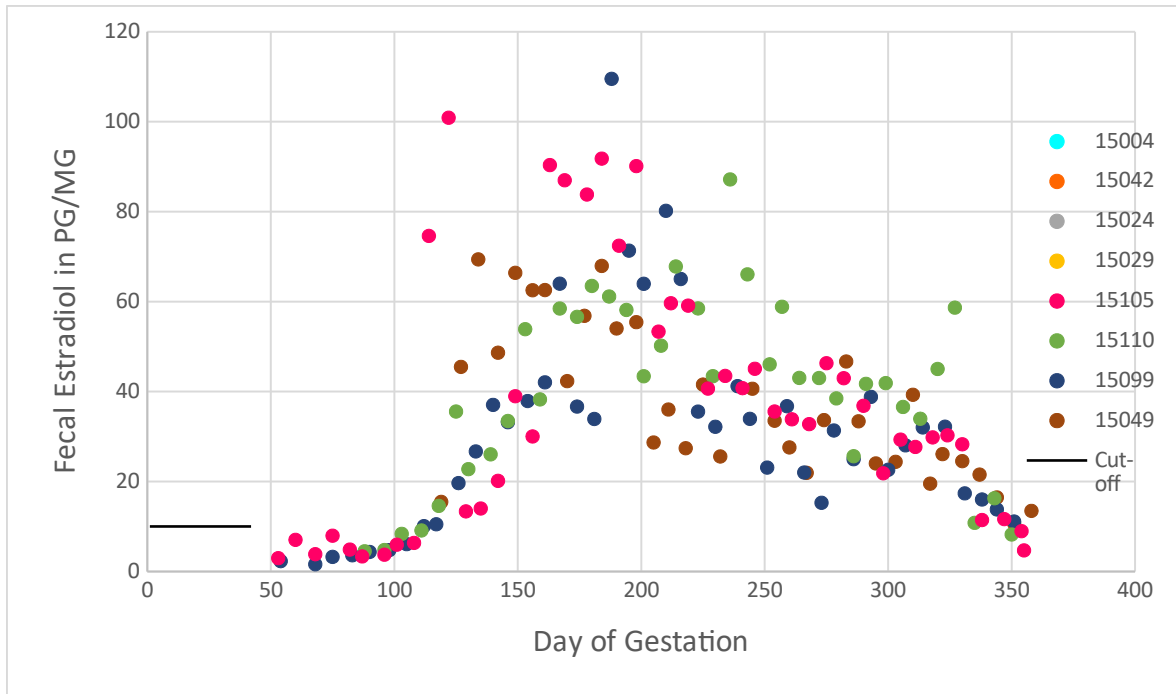


Figure 3A. Weekly fecal estradiol 17β concentrations in pg/mg of feces throughout gestation in 8 domestic mares, shown as numbers. Cut-off value is 10 pg/mg of feces and is indicated by black horizontal line.

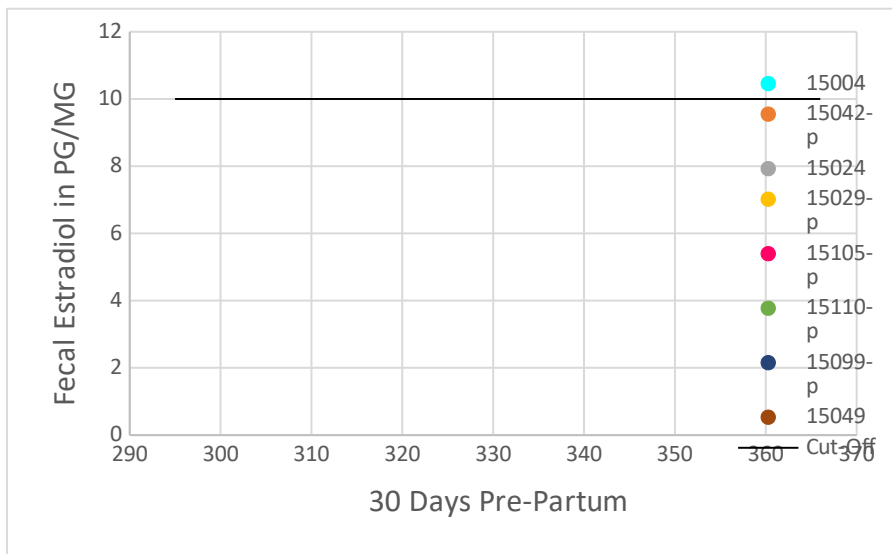


Figure 3B. Fecal estradiol 17β concentrations 30 days prior to parturition in 8 domestic mares. Cut-off value is 10 pg/mg of feces and is indicated by the black line. Mare numbers labeled with a -p indicate samples taken on the day of parturition.

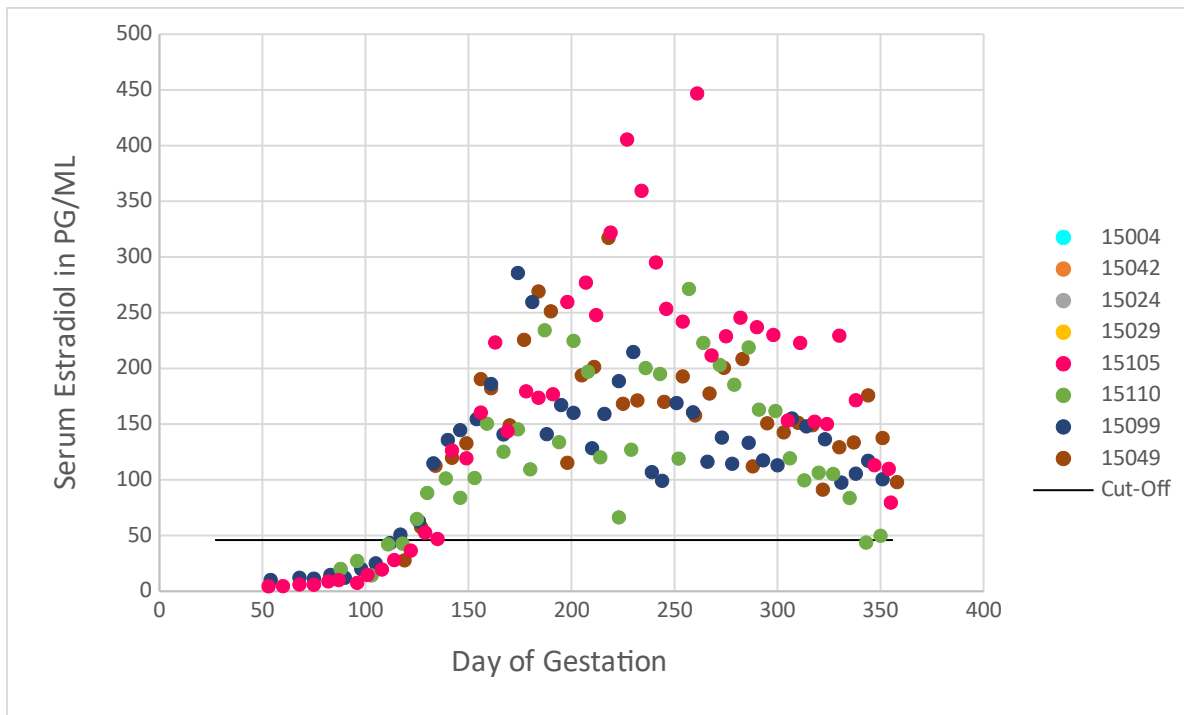


Figure 4A. Weekly serum estradiol 17 β concentrations in pg/mL of serum throughout gestation in 8 domestic mares. The cut-off value is 46 pg/mL of serum and is indicated by black horizontal line.

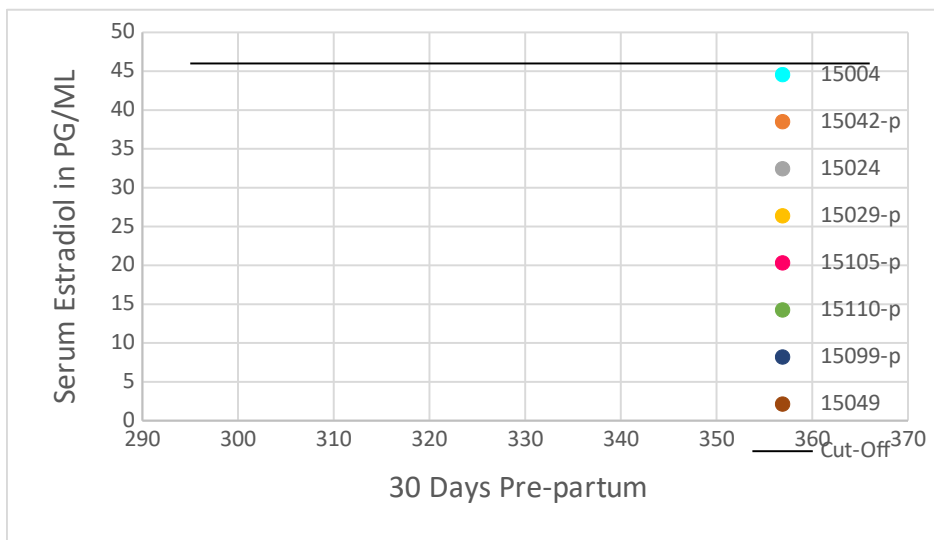


Figure 4B. Serum estradiol 17 β concentrations 30 days prior to parturition in 8 domestic mares, with the 46 pg/mL cut-off indicated by black line. Mare numbers labeled with a -p indicate samples taken on the day of parturition.

Study 2: Measurement of Fecal and Serum Estradiol in the Feral Mare

Objectives

- 1) to correlate findings of measured fecal and serum estradiol concentrations compared to ultrasound results
- 2) to determine the accuracy of pregnancy status from single fecal samples from feral mares.

Materials and Methods

This study was initiated in October 2009 with a park-wide roundup of all the horses in THRO, and included feral mares born between 1992- 2007. The first roundup included 48 feral mares, while the roundup in September 2013 included the initial 48, plus three more, for a study total of 51 mares. The 2009 roundup was the initiation of the GonaCon-Equine study, with 25 mares injected with GonaCon-Equine (treatment), and 23 mares injected with saline (control). In 2013, three additional mares were added to the study and assigned to the control group, for a total of 25 GonaCon-Equine treated mares, and 26 saline treated mares. Repeat GonaCon-Equine injections were completed at this time, as well as repeat saline injections to the original control group, and three new saline injections to the added three mares.

The process for fecal and serum collection from each mare was the same for both roundups. Once a mare was positioned in the squeeze shoot, a fecal grab was taken, followed by ultrasound to determine whether the mare was pregnant. While this was occurring, two red top vacutainer tubes of jugular blood were drawn using an 18-gauge 1.5-inch needle. All fecal samples collected at both roundups were placed in whirl packs labeled with date and mare identification, then frozen at -20°C until processing. All blood samples sat at room temperature for 30 minutes and were then centrifuged for 30 minutes at 2400xg, after which resulting serum was placed into 2 mL cryogenic vials and frozen at -20°C until extraction.

Following the 2009 round-up, yearly fecal sample collection was initiated for the 51 mares in the study. The breeding season in THRO ranges from March – August (Baker et al 2018), and aside from the roundup samples, the majority of the additional 272 fecal samples were collected during the month of November from 2010-2015. A smaller proportion of samples were also collected in February from 2011 – 2015. During each collection year, an attempt was made to obtain at least one fecal sample for each of the 51 mares on the study. Due to the large size and rough terrain of THRO, it was not always possible to find all mares every year. For this study, only samples obtained during the non-breeding season were used, with the rationale that no additional mares would be bred between the months of September – February. Fecal collection was completed by locating the mare of interest, and then tracking her until she defecated. Feces was collected from 4-5 areas within the pile; samples urinated on by the band stallion were not used. Samples were placed into whirl packs labeled with date and the mare's information, then placed into a cooler with ice packs. At the conclusion of each 8-12 hour collection day, samples were frozen at -20°C until extraction.

As all fecal samples were collected from mares of unknown pregnancy status, day of gestation was estimated for each sample. This calculation was completed using the foaling data for the following year and counting the days between sample collection and foaling date, with an estimated gestational length of 345 days for all mares on the study.

Extraction and Assay

The method used for extraction and assay of domestic mare samples was also utilized in this study.

Results

Objective 1: to correlate findings of measured fecal and serum estradiol concentrations compared to ultrasound results

At the 2009 and 2013 roundups, a total of 51 mares were processed for feces and blood collection, 48 in 2009 and all 51 in 2013. Of the 48 mares in 2009, 39 were determined pregnant by ultrasound, while 9 were

found to not be pregnant. In 2013, ultrasound showed that 41 mares were pregnant and 10 were not. At the 2009 roundup, mares were assigned as either treatment or control mares, so there were no GonaCon-Equine treatment effects noted, as this year was the initiation of the study. In the 2013 roundup, 5 of the non-pregnant mares were treatment mares, while 5 were control mares.

While 39 mares were pregnant in the 2009 round-up, fecal and serum samples from three mares were not included in pregnancy data, as they did not have a foal the following year, making gestation estimates impossible. Table 1 indicates the samples taken relative to both round-ups, with the three mares removed from the data for the 2009 round-up, which indicates 36 pregnant mares. For fecal estradiol, of the 77 total samples collected over the span of both round-ups, 62 of them surpassed the cut-off day (COD) of 105 days, with 60 of the 62 exceeding the cut-off concentration (COC) of 10 pg/mg of feces. Conversely, 15 of the 77 samples were estimated to be collected prior to the COD, with 13 of the 15 failing to surpass the COC. None of the samples from the non-pregnant mares were above the COC. Relative to serum estradiol, 49 of the 77 total samples surpassed the COD of 128 days, with 34 of the 49 samples exceeding the COC of 46 pg/mL of serum. Of the 28 samples estimated to be collected prior to the COD of 128, 26 of them failed to exceed the COC. As noted in feces, none of the non-pregnant mares had serum estradiol values that exceeded the COC. The fecal and serum samples collected from mares past the estimated COD that failed to surpass the COC are shown in Table 2, while Table 3 displays the data from the 19 non-pregnant mares.

The data described in Table 1 is shown graphically in Figures 5A, 5B, 6A and 6B. Figures 5A and 5B depict the fecal estradiol concentrations of pregnant mares for both roundups, for a total of 77 fecal samples. Figure 5A illustrates the entire sample set from all mares, while Figure 11B indicates fecal data starting at day 80 of gestation, or just under a month prior to the COD of 105 days, along with the fecal estradiol COC of 10 pg/mg of feces.

Figures 6A and 6B show the serum estradiol concentrations of pregnant mares for both roundups, again for a total of 77 serum samples. Figure 6A illustrates the entire sample set for all mares, while Figure 6B shows the serum data starting at day 105, or roughly a month prior to the cut-off value of 128 days, along with the serum estradiol COC of 46 pg/mg of feces.

Preliminary Considerations for Study 2: Objective 1

As expected, the feral mare data is not as clear as the domestic mare data. This is most likely due to the fact that all sample gestation date estimates were based on a pre-determined estimate of 345 days of gestation length in conjunction with foaling data, therefore some gestational data could be incorrect. This could contribute to samples that passed the COD, but not COC, such as the fecal and serum samples seen in Table 2. For example, it is possible that either of the two fecal samples in that table could have been taken earlier in gestation than calculated, and not actually beyond the COD. While this is plausible, in order to complete the calculations for the data, gestational length had to be chosen, and 345 days is the longest average gestation noted in the literature (Ginther 1992).

Aside from unavoidable potential error in calculations relative to gestational timing, the fecal and serum results obtained from feral mares across two roundups indicate that fecal estradiol is a reliable pregnancy determinant, while serum concentrations of estradiol fluctuate after the COD. This trend is similar to what was seen in pregnant domestic mare fecal and serum samples, in which 258/267 of fecal estradiol concentrations after day 105 and prior to two days of parturition remained above cut-off concentrations (Figure 3A). Although 239/252 serum samples collected after the COD of 128 surpassed the COC of 46 pg/mL, the remaining 13 fluctuated below the COC during mid-gestation, as seen in Figure 4A.

Table 1. Pregnant and non-pregnant feral mare fecal and serum estradiol samples that surpassed and missed cut-off values during the 2009 and 2013 roundups.

Roundup 2009	Roundup 2013	
Mares: 48 total	Mares: 51 total	
Pregnant: 36	Pregnant: 41	
Non-pregnant: 9	Non-pregnant: 10	
ALL MARE FECAL ESTRADIOL CONCENTRATIONS		
PREGNANT MARE TOTAL		
Fecal Estradiol	Cut-Off 105 days	Cut-Off: 10 pg/mg
Surpassed	62/77 samples	60/62 samples
Below	15/77 samples	13/15 samples
ALL NON-PREGNANT MARES	0/19 mares	0/19 mares
ALL MARE SERUM ESTRADIOL CONCENTRATIONS		
PREGNANT MARE TOTAL		
Serum Estradiol	Cut-Off 128 days	Cut-Off: 46 pg/mL
Surpassed	49/77 samples	34/49 samples
Below	28/77 samples	26/28 samples
ALL NON-PREGNANT MARES	0/19 mares	0/19 mares

Table 2. Roundup pregnant feral mare samples past the cut-off day, but not cut-off concentration.

Roundup Pregnant Mare Samples Below Concentration Cut-Off			
FECES		SERUM	
Estimated Day of Gestation	Fecal Estradiol in pg/mg	Estimated Day of Gestation	Serum Estradiol in pg/mL
110	5	129	13
115	8	130	32
		132	26
		132	26
		136	15
		141	44
		142	36
		143	45
		146	42
		153	23
		154	40
		156	39
		159	40
		165	26
		171	45

Table 3. Fecal and serum estradiol concentrations collected from non-pregnant feral mares during the roundups. The 2013 samples in red are from GonaCon-Equine treated mares. No sample concentrations surpassed feces COC of 10 pg/mg or serum COC of 46 pg/mL.

2009 Round-up		
Sample Date	Feces: PG/MG	Serum: PG/ML
10/11/09	1	6
10/11/09	1	5
10/11/09	1	2
10/11/09	1	5
10/11/09	1	8
10/11/09	1	2
10/11/09	2	1
10/11/09	4	13
10/11/09	1	9

2013 Round-up		
Sample Date	Feces: PG/MG	Serum: PG/ML
9/23/13	1	14
9/24/13	2	2
9/24/13	1	7
9/24/13	1	3
9/23/13	1	9
9/23/13	7	8
9/23/13	1	7
9/23/13	7	9
9/23/13	2	9
9/24/13	1	3

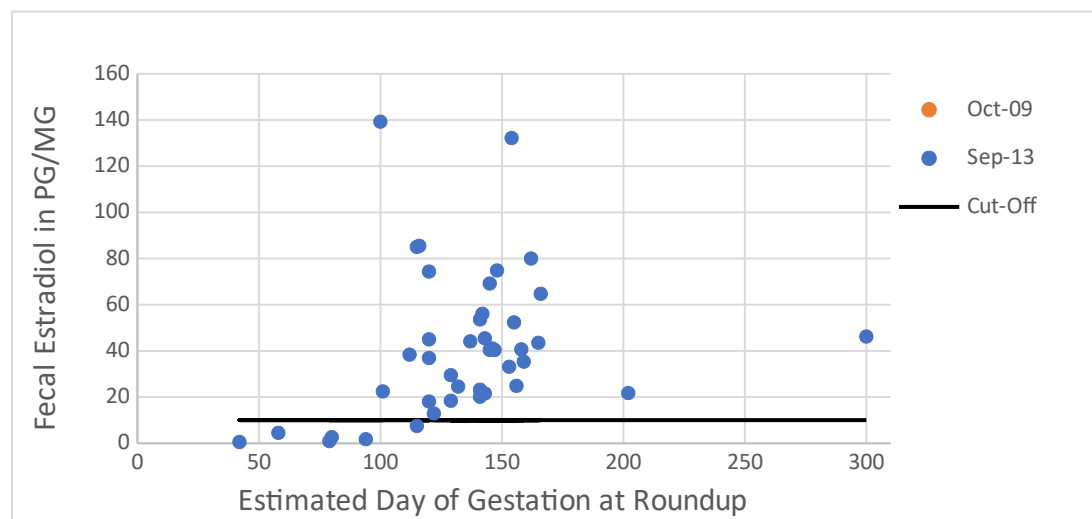


Figure 5A. Fecal estradiol 17β concentration from pregnant feral mare samples collected during the 2009 and 2013 roundups. The horizontal line indicates the cut-off concentration of 10 pg/mg feces.

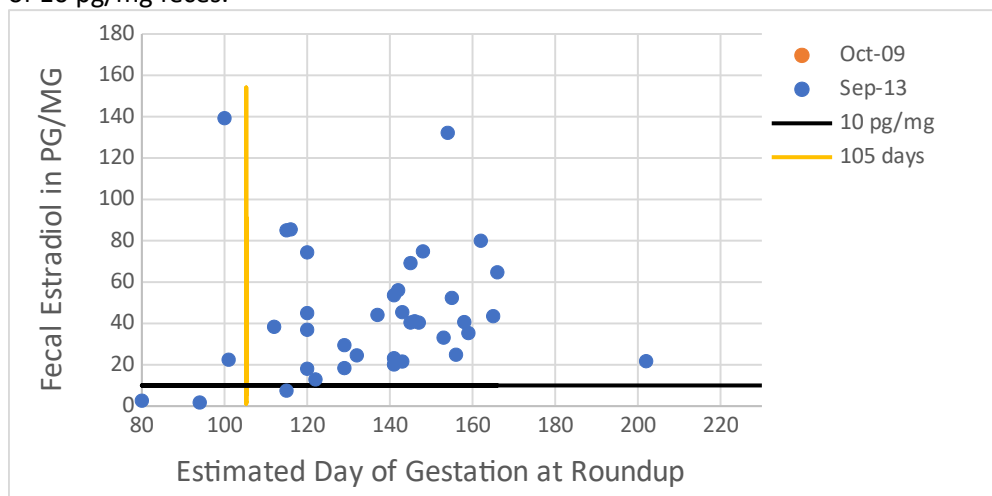


Figure 5B. Fecal estradiol 17β concentrations from pregnant feral mare samples collected after day 80 during the 2009 and 2013 roundups. The horizontal line indicates the 10 pg/mg cut-off concentration, while the vertical line indicates the cut-off day of 105.

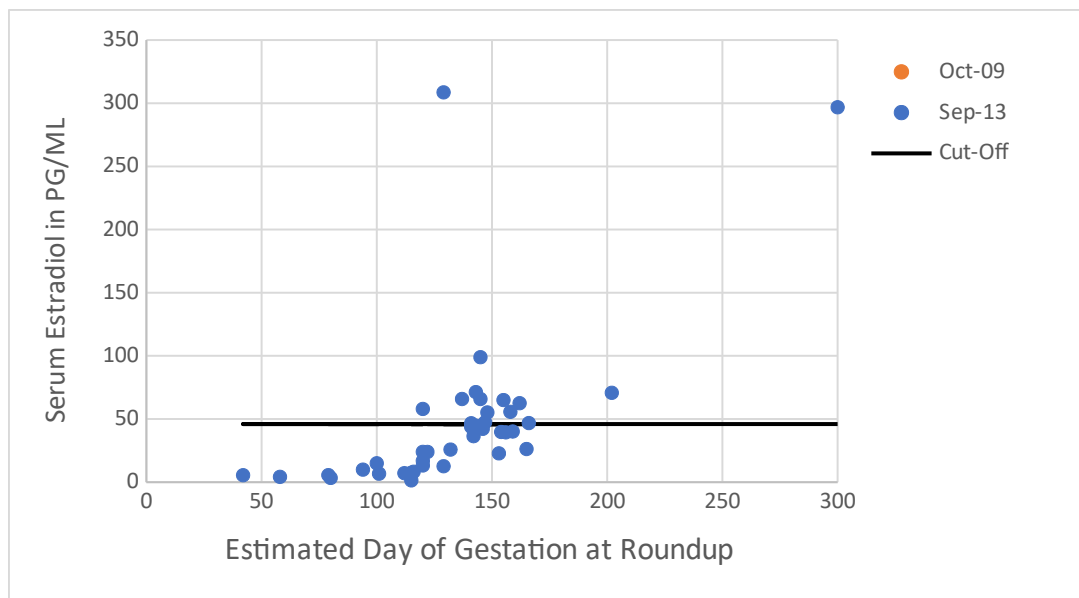


Figure 6A. Serum estradiol 17 β concentration from pregnant feral mare samples collected during the 2009 and 2013 roundups. The horizontal line indicates the cut-off concentration of 46 pg/mL serum.

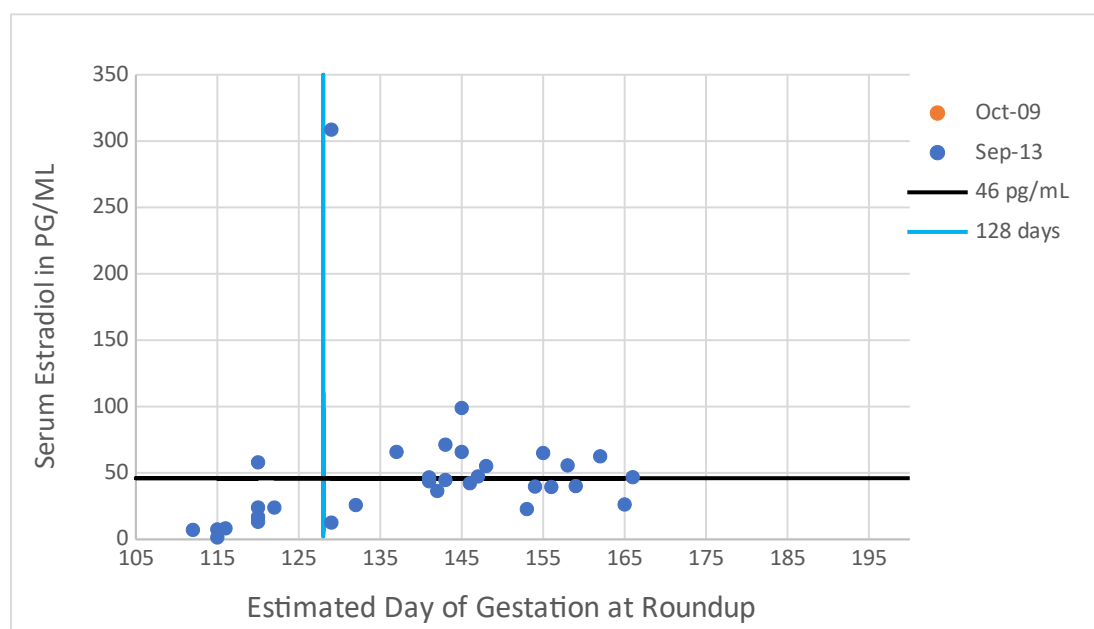


Figure 6B. Serum estradiol 17 β concentrations from pregnant feral mare samples collected after day 105 during the 2009 and 2013 roundups. The horizontal line indicates the 46 pg/mL cut-off concentration, while the vertical line indicates the cut-off day of 128.

Objective 2: to determine the accuracy of pregnancy status from single fecal samples from feral mares.

Although the roundups accounted for all serum samples, they only addressed a portion of the 368 total fecal samples collected during the study. Figures 7A-B, 8 and 9A-D show samples collected during both roundups, as well as November – February across 6 years of sampling. Figure 7A indicates the 121 samples from non-pregnant mares throughout the study, none of which surpass the COC of 10 pg/mg feces. Figure 7B indicates the same samples, but grouped according to treatment. The first grouping was from the 2009 roundup, which was the study initiation. Following that year, all additional samples taken from non-pregnant mares were denoted as either from treatment or control mares. Figure 8 illustrates the fecal concentration data for 225 pregnant mare samples. As it is difficult to discern the number of fecal samples from pregnant mares that failed to surpass the fecal estradiol COC in Figure 8, Figures 19A-D display a series of graphs divided into gestation

timepoints: 9A: 0-100 days of gestation ,9B: 101 – 151 days, 9C: 152 – 202, and 9D: 203 – parturition. Figure 9A depicts the first 100 days of gestation, where only 4 fecal estradiol concentrations surpassed COC of 10 pg/mg feces, all at day 100, with concentrations of 26, 36, 55, and 139 pg/mg feces. In Figure 9B (101-151 days of gestation), 5 samples failed to surpass the COC: 110 days; 5 pg/mg of feces, 111 days; 9 pg/mg feces, 115 days; 8 pg/mg feces, 118 days; 10 pg/mg feces, and 128 days; 9 pg/mg of feces. All samples from the subsequent two figures, 9C and 9D surpassed both COD as well as COC.

While the roundups occurred during September and October, the majority of the fecal collections during the 6-year sampling timeframe were completed in November. While Figure 9A-D shows the overall breakdown by sampling month relative to gestation, it is interesting to note the distribution of samples. Of the 41 samples taken in September, 35 of them surpassed the cut-off concentration of 10 pg/mg feces, while October resulted in 28/36, November in 126/132, and February 18/18. From this data, the overall percentage of samples over the cut-off value taken in September were 85.4%, 77.8% in October, 96.2% in November, and 100% in February. It should be noted that while these samples were taken over the course of six years, they were from 51 members of the entire population, and of the 226 samples discussed here, just under half were taken in November, resulting in an uneven distribution over the sampling timeframe.

Even with the uneven sampling distribution, the majority of samples taken in November were in the estimated 152-202 day range of gestation, with only 4/131 samples taken prior to the COD of 105. This would mean that in a population with a similar breeding timeframe to mares in Theodore Roosevelt National Park (THRO), samples taken in November would result in the majority being past 105 days of pregnancy, and more likely to be in the estimated 152-202 day range. When examining October samples, 9/36 were taken prior to 105 days, indicating roughly 25% of samples potentially taken too early in gestation to reliably distinguish between pregnant and non-pregnant individuals. Six samples taken in September were below the COD of 105 days, which would result in about 14.6% of samples taken too early. Although 100% of samples taken in February resulted in samples over 105 days, 14/18 samples were during days 203- parturition time point, which would be too late for making effective management decisions for feral herds. With that said, however, when only looking at the sample distribution relative to estimated 101-151 days, 96% of the September samples and 91.7% of both the October and November samples were above the COC. Knowing this, it would therefore be up to the managers of individual feral herds to determine what level of uncertainty in sample concentration returns they would be comfortable with.

As mentioned previously, in the 2009 round-up, there were three mares who were pregnant at the time of the round-up, but no foals were found the following year. Aside from these three, there were an additional 19 mares throughout the study whose fecal estradiol values indicated pregnancy at time of sampling, but no foals were found. Data for the three mares from the 2009 round-up is shown in Table 4, while Figure 10 illustrates the data from the remaining 19.

Preliminary Considerations for Study 2: Objective 2

Determining pregnancy status from single fecal samples taken over the course of the study was found to be possible when measuring fecal estradiol from mares of this herd. From the data collected during the study timeframe, the overall percentage of samples over the cut-off value taken in September were 85.4%, 77.8% in October, 96.2% in November, and 100% in February. It should be noted that while these samples were taken over the course of six years, they were from 51 members of the entire population, and of the 226 samples discussed here, just under half were taken in November, resulting in an uneven distribution over the sampling timeframe.

Even with the uneven sampling distribution, most samples taken in November were in the estimated 152-202 day range of gestation, with only 4/131 samples taken prior to the COD of 105. This would mean that in a population with a similar breeding timeframe to mares in Theodore Roosevelt National Park (THRO), samples

taken in November would result in the majority being past 105 days of pregnancy, and more likely to be in the estimated 152-202 day range. When examining October samples, 9/36 were taken prior to 105 days, indicating roughly 25% of samples potentially taken too early in gestation to reliably distinguish between pregnant and non-pregnant individuals. Six samples taken in September were below the COD of 105 days, which would result in about 14.6% of samples taken too early. Although 100% of samples taken in February resulted in samples over 105 days, 14/18 samples were during days 203- parturition time point, which would be too late for making effective management decisions for feral herds. With that said, however, when only looking at the sample distribution relative to estimated 101-151 days, 96% of the September samples and 91.7% of both the October and November samples were above the COC. Knowing this, it would therefore be up to the managers of individual feral herds to determine what level of uncertainty in sample concentration returns they would be comfortable with.

Prior to this study, foaling rates in the park were determined by the number of live foals seen on the ground. Over the course of this study, there were a total of 22 pregnant mares whose foals the following year were not seen. Of the 22, 3 of the mares were part of the 2009 round-up, so were known to be pregnant, but no foals were seen the following year. The serum and estradiol concentrations for these mares are in Table 4, and all but one surpassed respective fecal and serum concentration cut-offs. While the second serum sample in the table of 32 pg/mL failed to surpass the COC, the corresponding fecal sample concentration of 38 pg/mg feces did, indicating potential early pregnancy. Figure 10 depicts the fecal concentrations of these three mares along with the remaining 19, whose fecal samples were taken not knowing pregnancy status. As can be seen in this figure, all fecal estradiol concentrations are above the cut-off value of 10 pg/mg, indicating that all 22 mares should have been at least 105 days into gestation. This information is useful because if THRO continues to take fecal samples from mares in the fall, the use of foaling rates won't need to be utilized, as pregnancy status relative to fecal estradiol measurement will be known.

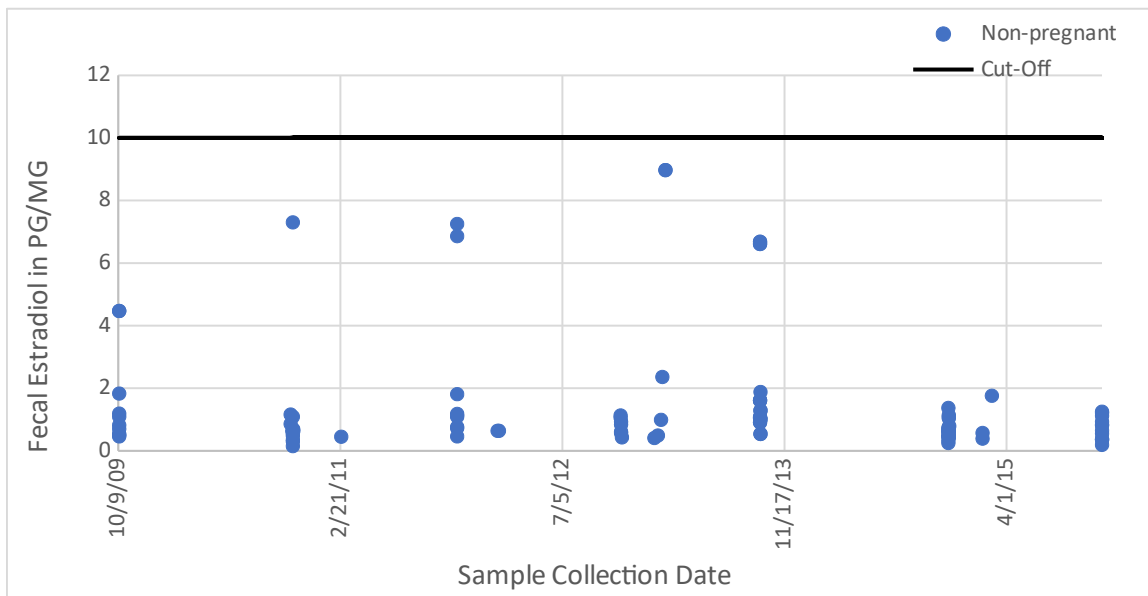


Figure 7A. Total distribution of fecal estradiol concentrations in pg/mg from collected samples of non-pregnant feral mares. The horizontal line indicates the cut-off concentration of 10 pg/mg feces.

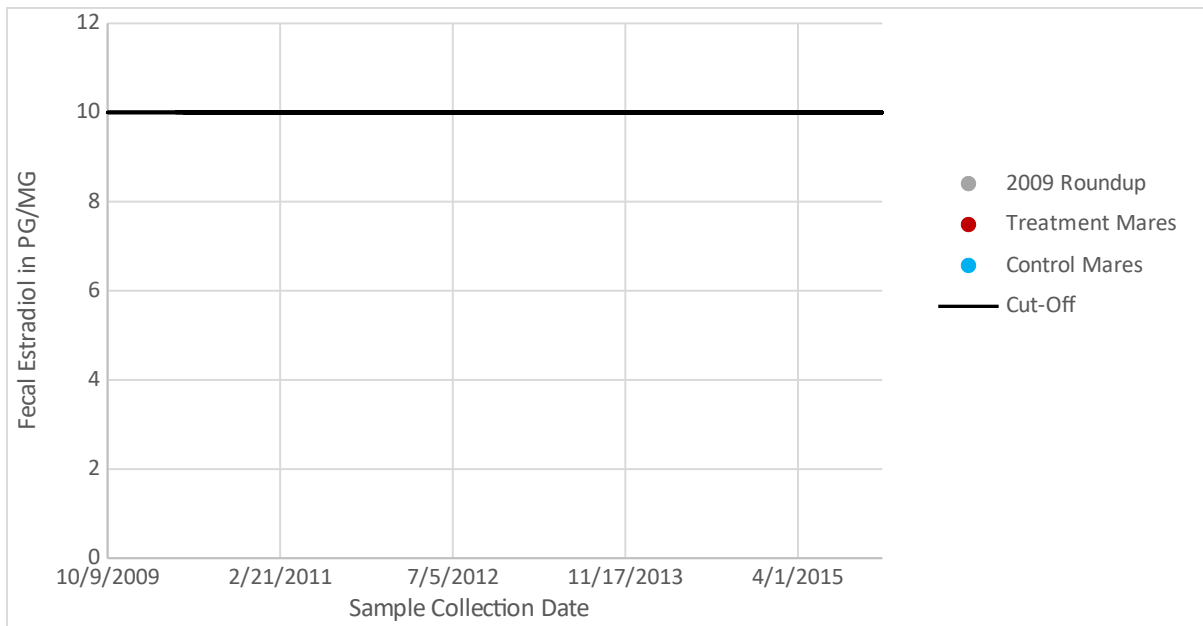


Figure 7B. Fecal estradiol concentrations in pg/mg from collected samples of non-pregnant feral mares. Samples are delineated as those collected at the 2009 roundup, or start of the study, as well as during proceeding years, labeled as treatment and control mare samples. The horizontal line represents the 10 pg/mg cut-off concentration.

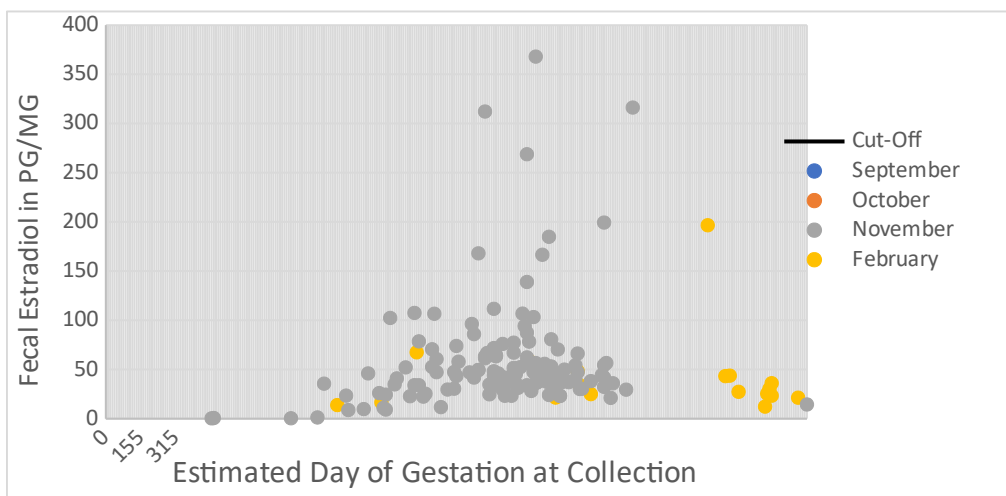


Figure 8. Fecal estradiol concentrations from samples collected from pregnant feral mares in pg/mg. The horizontal line indicates the COC of 10 pg/mg feces.

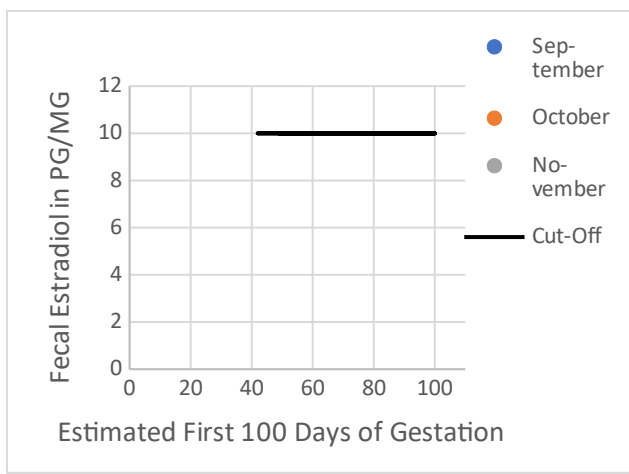


Figure 9A

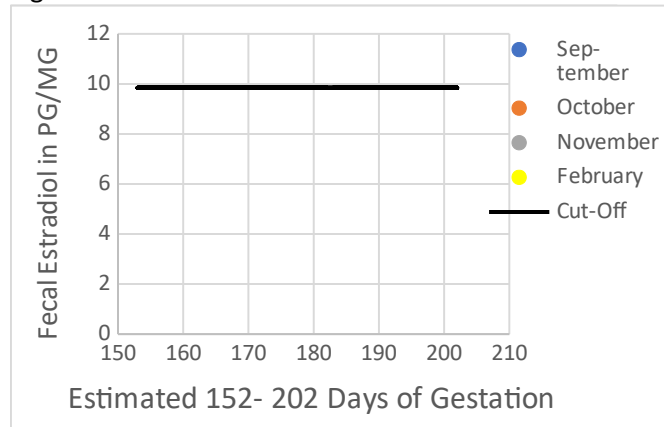


Figure 9B

Figure 9C

Figure 9D

Figure 9A-D. Progression of estimated day of gestation relative to fecal estradiol concentration in pg/mg. All horizontal lines indicate the cut-off concentration of 10 pg/mg feces.

Table 4. Fecal and serum concentrations taken from 3 pregnant feral mares at roundups whose foals were not found the following year.

Date of Collection	Fecal Estradiol in pg/mg	Serum Estradiol in pg/mL
10/11/09	19	52
10/11/09	38	32
10/11/09	35	63

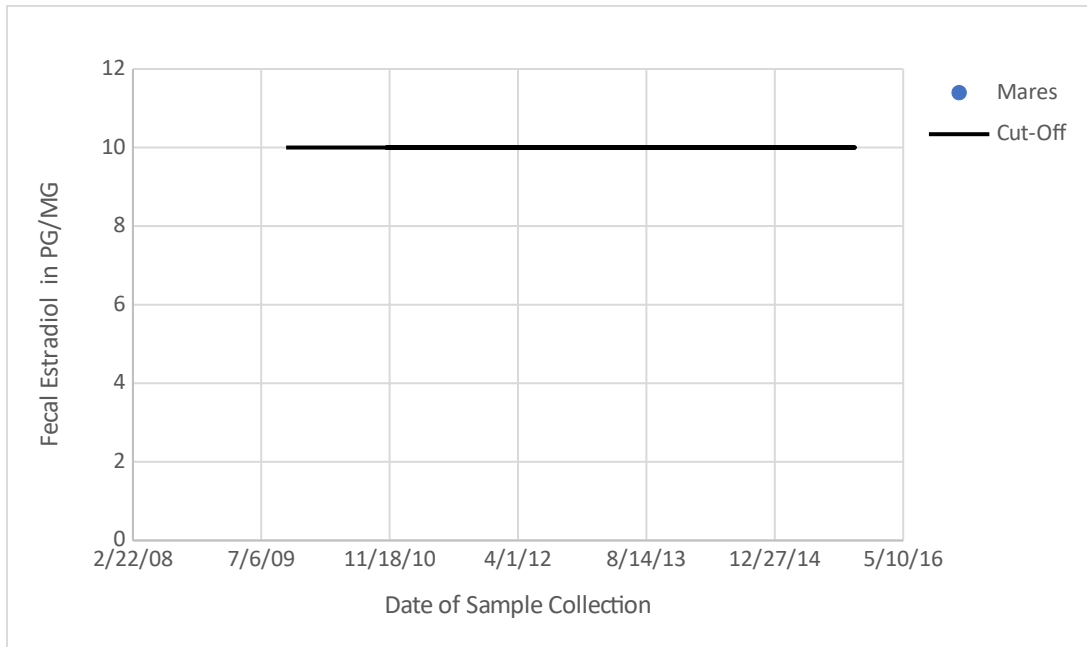


Figure 10. Fecal estradiol concentrations from 22 feral mares who were pregnant at time of collection, but whose foals were not found the following year. The horizontal line indicates the concentration cut-off of 10 pg/mg feces.

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