THESIS

USE OF FECAL AND SERUM ESTRADIOL ANALYSIS FOR ESTIMATION OF PREGNANCY STATUS IN THE MARE

Submitted by

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ABSTRACT

USE OF FECAL AND SERUM ESTRADIOL ANALYSIS
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Overabundant feral horse populations within the United States cause significant and detrimental economic and ecological impacts. Aside from helicopter roundups and long-term holding facilities, current management practices of feral horses include application of contraception in conjunction with non-invasive determination of pregnancy through the measurement of fecal steroid metabolite monitoring. Prior to this study, the earliest timing of definitive pregnancy diagnosis was between 120 – 180 days of gestation, when measuring total unconjugated fecal estrogens (Bamberg et al 1984; Kirkpatrick et al 1989), or from samples taken at least 150 days of gestation when measuring fecal estrone sulfate (Henderson et al 1998 and 1999). The studies in this thesis examined measurement of estradiol 17β, an estrogen that has yet to be quantified in the feces of domestic and feral mares. The overall objectives of the studies in this thesis were to determine the efficacy of fecal and serum estradiol measurement in the estimation of pregnancy in the mare, as well as the definitive timing within gestation when fecal and serum concentrations diverged from those of non-pregnant mares.

The first study of this thesis utilized 8 pregnant domestic mares with known embryo transfer dates, as well as 8 non-pregnant cycling mares. Weekly fecal and blood samples were collected from the pregnant mares for the entirety of gestation, while daily fecal and blood samples were taken from the cycling mares for 23 days. Radioimmunoassay (RIA) specific for estradiol 17β was used to quantify extracted fecal and serum samples for the two groups. It was found that at a mean of 105 days of gestation, fecal estradiol concentrations in pregnant mares surpassed non-pregnant mare concentrations, with a calculated cut-off value of 10 pg/mg feces. Serum estradiol concentrations of

pregnant mares surpassed those of non-pregnant mares at an average of 128 days of gestation, with a concentration of at least 46 pg/mL serum. Additionally, aside from increasing earlier in gestation, compared to serum, fecal estradiol was found to fluctuate less throughout pregnancy.

The second study of this thesis examined 77 fecal and serum samples collected from 51 feral mares during two roundups in Theodore Roosevelt National Park (THRO), as well as 272 individual fecal samples collected over a 6 year period from the same 51 mares. Using the cut-off days and concentrations affiliated with the first study, correlative comparisons were made for the feral mare samples, and pregnancy status was elucidated. Of the 62 fecal samples taken during the roundups past the cut-off day of 105 days, 60 of them surpassed the fecal cut-off concentration of 10 pg/mg feces. Thirty-four of 49 serum samples taken past the cut-off day of 128 surpassed the cut-off concentration of 46 pg/mL. While only two of the 62 fecal samples taken past the cut-off of 105 days were below the cut-off concentration, 14 of the 49 serum samples taken past cut-off day 128 were below the serum cut-off concentration of 46 pg/mL serum. This trend was similar to what was seen in domestic mares.

Although the majority of the field fecal samples were collected in November, there were also the fecal samples from the September and October roundups, as well as a few February samples. While all but 4/131 samples from November were in the estimated 152 -202 day range of gestation, 6/41 in September, and 9/36 in October were below cut-off day 105. In a population similar to THRO, this could potentially result in 14.6% and 25% of concentrations from samples taken in September and October being too low to differentiate between pregnant and non-pregnant individuals. However, when examining the estimated sample distribution range of 101-151 days, 96% of September samples, 91.7% of both October and November samples resulted in concentrations above the cut-off value of 10 pg/mg.

From the studies completed in both domestic and feral mares, it can be said with confidence that the quantification of estradiol 17β using RIA is a reliable method for indicating pregnancy status in

the mare. Mares with fecal estradiol concentrations above 10 pg/mg from samples taken at least 105 days post conception were pregnant, as were mares with measured serum estradiol concentrations above 46 pg/mL collected after 128 days post conception. Additionally, fecal samples taken from feral mares during the non-breeding season in THRO resulted in 96% of samples collected in September, and 91.7% of samples collected in October and November resulting in concentrations above the cut-off concentration of 10 pg/mg feces. This data supports the reliability of fecal estradiol measurement as a tool for pregnancy status determination in the mare.

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I would like to leave this note of acknowledgement with a phrase gifted to me from my father, one that I remind myself of daily: $- = \infty$. A reminder that a single line on a page has the potential to create anything.

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INTRODUCTION

Overabundant feral horse populations within the United States cause significant and detrimental economic and ecologic impacts. The lack of natural predators of the feral horse has resulted in populations increasing as much as 20% a year (National Research Council 2013). In areas with rapidly expanding feral horse numbers comes depletion of resources, which lead to starvation, dehydration, and death (BLM 2017). Additionally, increased herd numbers result in compounded stress on native plant communities, specifically because horses are indiscriminate grazers, causing fewer plant species to remain ungrazed (Beever 2003; Zeigenfaus et al 2014). Reduction of native grasses results in increased growth of non-palatable and invasive plant species such as cheat grass and bitterbrush, lowering the nutritional value of available forage for horses, as well as other species sharing the same range (Beever and Brussard 2000; Davies and Boyd 2019). Feral horses also vie with native wildlife for accessible resources, and because of their large size and aggressive behavior (Berger 1985), they are often able to out compete smaller, native ungulates such as deer, pronghorn, and bighorn sheep from necessary resources such as water (Berger 1985; Coates and Schemnitz 1994).

The Bureau of Land Management (BLM) has been tasked with the management of feral horses on public land, which includes ensuring sufficient grazing capacity for both native and non-native species. To do this, the BLM developed Appropriate Management Levels (AMLs), which have specific Herd Management Areas (HMAs). AMLs reflect the calculated numbers of feral horses allowable within each HMA, while considering ecological factors including native wildlife, recreation, and livestock grazing (Davies and Boyd 2019). As of March 1st, 2020, the overall AML was calculated to be sustainable at 23,851 horses, while the actual estimated horse population was approximately 79,600, an excess of nearly 56,000 individuals (BLM 2020).

Due to the significant overpopulation, the BLM oversees annual roundups, but dependent on overpopulation levels within a state and associated HMA, there are oftentimes more than one yearly gather. These gathers have resulted in the roughly 46,000 horses currently being held in off range facilities, with cumulative management costs expected to exceed $1 billion in the next 20 years (BLM 2017).

Aside from populations managed by the BLM, the National Park Service (NPS) is responsible for overseeing approximately 1000 feral horses across 20 NPS units. Horses within the NPS system fall into a variety of categories, including minimal management of populations not considered to be of cultural importance, up to significant intervention and management of herds considered to be vital to the cultural landscape of the park (Powers 2014). Horses within the NPS system, as well as throughout the BLM, are managed with non-lethal fertility control, such as immunocontraceptive drugs. As managers of feral horse populations make decisions based on pregnancy status of individuals within their populations, knowledge of pregnancy status is vital.

While pregnancy determination in domestic mares is feasible through blood draws measuring reproductive hormones, such as equine chorionic gonadotropin (eCG) and estradiol, bleeding feral horses is not feasible without restraint. Accordingly, many managers have opted for the measurement of fecal steroids. Historically, the most commonly measured of the fecal estrogen metabolites is estrone sulfate, with a variety of studies (Linklater et al 2000; Henderson et al 1998 and 1999) accurately determining pregnancy status in feral mares with samples taken at least 150 days into gestation. Other studies measuring total unconjugated fecal estrogens have determined definitive pregnancy status at approximately 120-180 days of gestation. No studies in the literature have resulted in definitive pregnancy status through measurement of fecal estrogens earlier than 120 days of gestation.

The first study presented in this thesis examines the determination of a reliable cut-off day and estradiol concentration that clearly delineate between pregnant and non-pregnant domestic mares, specific to feces and serum. The second study correlates the cut-off values found in domestic mares to those in feral mares. Feral mare samples measured in this study were fecal and serum samples taken during two roundups, as well as single fecal samples collected over the course of six years.

CHAPTER 1

Review of Literature

History of Feral Horses

The diverse background of the feral horse has led to overabundance within the United States, causing significant detrimental ecological and economic impacts. Truly wild horses, or horses without domesticated ancestry, went extinct in North America at the end of the Pleistocene era between 10,000 and 14,000 years ago (Grayson 2006; Davis and Boyd 2019). In the sixteenth and seventeenth centuries, self-sustaining feral horse herds were introduced by Spanish explorers (Haines 1938), and in the proceeding years, the numbers only continued to rise. This was due in part to increased lifespan resulting from decreases in predation, increased mobility due to lack of confinement, and both accidental and intentional release of domestic horses to the range (Beever 2003). Additionally, as feral horse band structure is generally one stallion with multiple mares, ranchers took advantage of feral herds, killing band stallions and then artificially manipulating blood lines and altering genetics to their liking by releasing domestic stallions into the herds. (Bowling 1994; Hyslop 2017).

Feral herds continued to increase in the United States until the mid-nineteenth century, leading to an estimated population peak of 2-7 million animals (Ryden 1978), followed by a sharp decline throughout the mid-20th century. This was in part due to the Taylor Grazing Act, which initiated grazing districts in which land was apportioned, resulting in feral horse removal, as well as re-domestication (Wagner 1983). By the early 1970's there was an estimated 17,300 feral horses on public lands within the United States (BLM 2020). To protect declining feral horse (and burro) herds, the Wild Free-Roaming Horses and Burro Act (WHBA) was passed in 1971; stating that "wild, free-roaming horses and burros shall be protected from capture, branding, harassment or death; and to accomplish this, they are to be

considered in the area where presently found, as an integral part of the natural system of the public lands." (BLM 2020).

Feral Horse Management

At the signing of the WHBA, the Bureau of Land Management (BLM) was charged with the protection and management of these herds. To ecologically maintain public lands, ensuring sufficient grazing resources for both native and non-native wildlife, the BLM produced annual Appropriate Management Levels (AMLs), and within these, specific Herd Management Areas (HMAs), totaling 26.9 million acres across ten states (Figure 1) (BLM 2020). HMAs are areas where feral horses and burros existed at the signing of the WHBA and have since been designated as areas of continued management by the BLM (BLM 2017). AMLs reflect the calculated numbers of horses and burros allowable within each HMA, while considering ecological factors including native wildlife, recreation, and livestock grazing (Davies and Boyd 2019). As of March 1st, 2020, nearly 50 years after the signing of the WHBA, the overall AML was calculated to be 23,851 feral horses, while the actual estimated horse population was 79,600, an excess of nearly 56,000 individuals (BLM 2020); of the 177 HMAs, nearly 80% of them are over their projected AML (BLM 2020).

With the current equine overpopulation comes a myriad of problems, most of which are ecologically associated with rangeland issues and interactions. Due to a lack of natural predators, most feral horse populations have the capacity to reach mean annual increases of 20% (Nat Research Council of the National Academies 2013). With herd size increases in areas of already strained ecosystems, forage and water resources become depleted, resulting in starvation, dehydration, and death (BLM 2017). Additionally, increased herd numbers result in compounded stress on native plant communities, specifically because horses are indiscriminate grazers, causing fewer plant species to remain un-grazed compared to areas of grazing by other ungulates (Beever 2003; Zeigenfuss et al 2014).

Figure 1. Herd Management Areas (HMAs), Bureau of Land Management. Blue areas are horse HMAs, orange are burro (BLM 2020).

Due to overgrazing, areas with feral horses have reduced plant cover of native plants, as well as increases in non-palatable and invasive plant species such as cheat grass and bitterbrush (Beever and Brussard 2000; BLM 17; Davies and Boyd 2019;). Aside from botanical impacts, feral horses vie with native wildlife for resources, and due to their larger size and oftentimes aggressive behavior (Berger 1985), are able to successfully displace other ungulates such as deer, bighorn sheep and pronghorn away from necessary resources such as water (Berger 1985; Coates and Schemnitz 1994; Gooch et al 2017).

With the intention of decreasing overabundant feral horse herds in a humane manner, the BLM currently manages multiple herds across ten states, each of which has its own affiliated HMA and corresponding AML with calculated capacities. The BLM is allotted an annual monetary appropriation, and in fiscal year (FY) 2019, received $80.6 million, of which $58 million was utilized for off range handling and holding facilities of horses collected via helicopter round-ups, an additional expenditure of $4 million annually (BLM 2020). Depending on the state and HMA, there are oftentimes more than one annual gather, resulting in roughly 46,000 horses currently being held in off range facilities (BLM 2020), with expected cumulative costs to exceed $1 billion over the next 20 years (BLM 2017). Although the BLM attempts to mitigate numbers via horse and burro adoptions to the public, even in years with record-breaking numbers, such as in FY2019 with 7104 adoptions (BLM 2020), the sobering fact remains that thousands of horses and burros remain in long term holding facilities, frequently for life.

While the BLM is tasked with feral horse management on public lands, they do not care for hoses that reside within national parks, which are under the management of the National Park Service (NPS). Upon its' establishment in 1916, the NPS's primary mission has been: "…to conserve the scenery and the natural and historic objects and the wild life therein and to provide for the enjoyment of the same in such manner and by such means as will leave them unimpaired for the enjoyment of future generations." (NPS 2006). Additionally, the NPS management policies remind managers that "All exotic

[non-native] plant and animal species that are not maintained to meet an identified park purpose will be managed – up to and including eradication – if 1) control is prudent and feasible, and 2) the exotic species interferes with natural processes, native species, or natural habitats….." (NPS 2006). As feral horses are considered non-native species by the NPS, it therefore depends on each individual park, and the unique circumstances surrounding each herd as to management protocol.

Feral horses currently reside in approximately 20 NPS units across the United States, and while specific numbers are unknown, estimates are of about 1000 individuals (Powers 2014). These horses fall into one of four management categories; the first of which the animals are residents within the NPS unit, but not specifically maintained as a cultural resource. In this situation, populations have existed prior to the park establishment, and management ranges from eradication, to nothing at all, due to lack of funding (Powers 2014). The second category occurs when animals have trespassed onto park lands, resulting in roundups for removal and adoption, while the third consists of trespass livestock from privately owned property. In this case, determination of ownership is attempted, and if not established, animals are considered abandoned and removed, sometimes through lethal means (Powers 2014). The last category is both the most popular and controversial, as it includes non-native horses and burros that are included as part of the cultural landscape of the park; the herds tend to be small, highly visible, and include many interested stakeholders in the well-being and management of the animals (Powers 2014).

Areas in which horses are maintained as culturally important have made significant research contributions within the field of non-lethal fertility control as a means of population management (Powers 2014). This form of management is ideal for feral equines and could contribute to decreasing the numbers of horses sent to and housed in the BLM long term holding facilities. In addition to the use of immunocontraception, the combined utilization of pregnancy determination through non-invasive means as early as possible in gestation would be helpful in the overall management of feral horse herds.

Management measures such as these would be dependent on reproductive factors such as cyclicity of mares within the population, and relative hormone profiles.

Reproduction in the Mare: Cyclicity

The mare is a seasonally polyestrous animal, undergoing estrous cycles from April through September in the Northern Hemisphere (Nagy et al 2000). Each cycle averages 21-22 days in length, and is divided into a follicular phase (estrus), in which the mare is sexually receptive, and a luteal phase (diestrus), in which she is not. Estrus generally lasts for 4-7 days of the cycle, with diestrus comprising the rest at 10-15 days (Figure 2) (Brinsko et al 2011). This pattern of estrous cyclicity is maintained by a specific hormone balance of hypothalamic, pituitary, and gonadal hormones; the effector of which is gonadotropin releasing hormone, or GnRH.

Responsible for reproductive function, GnRH is a highly conserved decapeptide found in all vertebrate mammalian species (Ginther 1992). Produced by the neurosecretory cells of the hypothalamus, its pulsatile secretion directly impacts the release of gonadotrophins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) from anterior pituitary gonadotropes. The release of GnRH is directly related to photoperiod. During the anovulatory season, hypothalamic GnRH content and secretion is reduced (Bergfelt and Ginther 1991; Peltier et al 1997). The absence of reproductivity in the fall and winter is linked to an inverse relationship between shorter photoperiods and GnRH secretion stemming from inhibitory neuronal interactions of the pineal gland and hypothalamus (Ginther et al 1992; Nagy et al 2000). Although mares have been observed exhibiting estrus-like behavior during the anestrous season, they are incapable of reproduction, as ovulation does not occur (Nagy et al 2000).

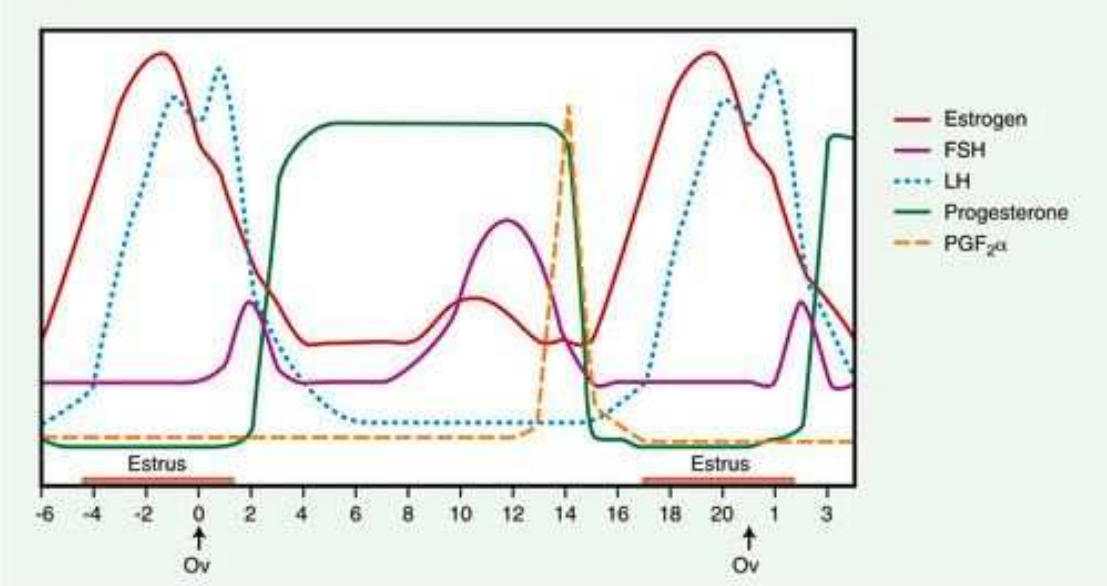


Figure 2. Estrous cycle of the mare. (Brinsko et al 2011)

In the mare, the anovulatory season has been divided into three periods: transition into the fall months, in which the hypothalamic-pituitary- gonadal (hpg) axis activity begins to decline, deep anestrus of the winter months, where there is little hpg axis activity, and transition into the spring, in which increasing day length causes a gradual recrudescence of the hpg axis, allowing for increasing follicular growth and eventual ovulation (Donadeu and Watson 2006). Follicular growth in the mare is mediated via follicular waves; classified as either minor or major, with first emergence of minor waves during the spring transition, producing non-ovulatory follicles (Donadeu and Ginther 2000; Donadeu and Watson 2007; Donadeu and Pederson 2008;). Major waves, occurring towards the end of diestrus, result in the production of the dominant, ovulatory follicle (Donadeu and Pederson 2008).

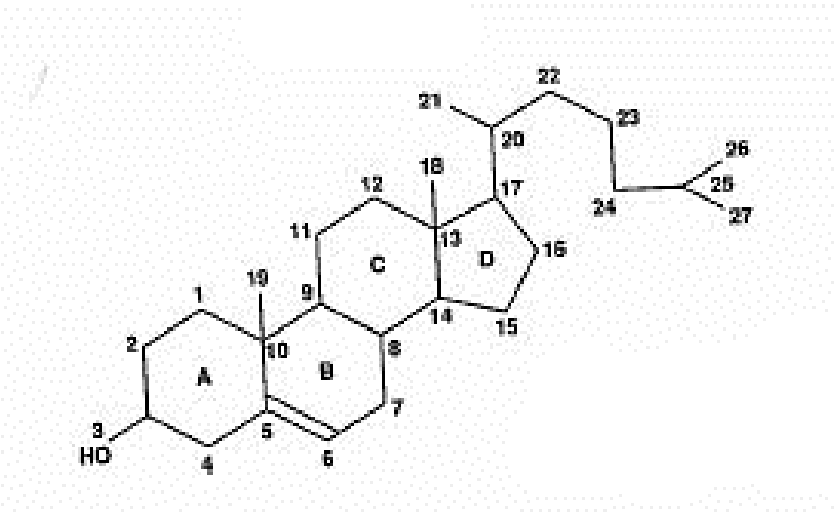
Unlike pre-antral follicles, antral follicles cannot continue maturation without adequate gonadotropin stimulation. The process of follicular growth is mediated by FSH levels and characterized by the periodic growth of cohorts, or follicular waves (Bergfelt and Ginther 1991). Preceded by increasing levels of FSH from the anterior pituitary gland, equine follicular waves involve the near simultaneous growth of follicles at a common rate until deviation, or follicular selection, which occurs when the two largest follicles of the cohort reach approximately 22 mm in diameter (Donadeu and Ginther 2002). While the wave-preceding increase of FSH is important in supplementing follicular growth, the ability of follicles to reach ovulatory diameter is dependent on LH, and without it, they subsequently lose the ability to do so (Ginther 1992). Deviation occurs roughly 7 days prior to ovulation and is indicated by continuous growth of the largest follicles, as well as concurrent increases in LH, inhibin, and estradiol. With the increasing release of inhibin from the dominant follicle, subordinate follicles no longer receive enough FSH to continue to grow, and begin to regress and undergo atresia (Donadeu and Pederson 2008). Once deviation occurs, the dominant follicle continues to grow until reaching an ovulatory diameter of 30-45 mm, after which it will either ovulate or regress (Ginther 1992; Donadeu and Pederson 2008).

Ovulation is dependent on the magnitude of the LH surge, which is regulated by the availability of sufficient levels of estradiol (Donadeu and Pederson 2008). Following ovulation, the formation of the corpus luteum (CL) and resulting progesterone production causes negative feedback on LH at the level of hypothalamus and anterior pituitary. If the mare does not become pregnant, the CL undergoes regression, and initiation of the next follicular phase occurs (Brinsko et al 2011). Knowledge of the equine's seasonality relating to estrous cyclicity is key when determining practical functionality of monitoring hormone metabolites for determination of pregnancy status in the mare.

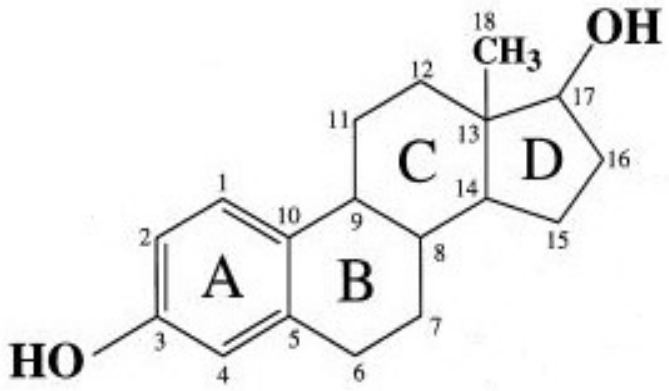
Measurement of Hormone Metabolites

Monitoring of hormone metabolites was originally developed in zoos because of the need to obtain biological samples without incurring capture and restraint stress (Lasley et al 1991). Initial work was focused on method development in monomorphic avians such as parrots. Sex and breeding potential were determined through measurement of unconjugated excrement steroids, specifically the ratio of estrogens and androgens (Lasley et al 1991).

The production of estradiol is ubiquitous among species. As shown in Figure 3, as a derivative of cholesterol, it has the same basic sterol structure of four rings (A-D), differing in the attached groups and double-bond placement (Ginther 1992). During estrogen metabolism, both estradiol and its metabolite estrone can undergo conjugation to either sulfates or glucuronides, the two most abundant circulating estrogen conjugates (Raftogianis et al 2000), a process which occurs in the liver of mammals (Kirkpatrick et al 1989). While estrogens can be measured in the urine, feces or blood in unconjugated or conjugated form, most early mammalian work focused on urinary concentrations of estrone conjugates, including estrone sulfates and glucuronides, which were measured to characterize estrous cycles, reproductive seasonality, and breeding potential in a variety of captive species (Lasley et al 1991).



A. Steric structure of cholesterol (adapted from Ginther 1992)



B. Steric structure of estradiol (adapted from Zhu et al 1998)

Figure 3. Steric structures of cholesterol and estradiol

Species included in early work were okapi (*Okapi johnstoni*), giraffe (*Girrafa Camelopardalis*), Indian rhinoceros (*Rhinoceros unicornis*), Asian elephant (*Elephas maximus*), lion-tailed macaque (*Macaca silenus*), and ruffed lemur (*Lemur variegatus*) (Lasley et al 1991).

As pregnancy detection is also vital in zoo-animal management, steroid metabolite monitoring for this purpose began to emerge. 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, accurate pregnancy determination has been accomplished using urinary estrone sulfate measurement in gorilla (*Gorilla gorilla*), tapirs (*Tapirus terrestris and T.indicus*), and Hartmann's zebra (*E.zebra*) (Lasley et al 1991). Although measurement of urinary estrogens have been effective, collection of urine is not always feasible, and fecal steroid metabolites have also been measured in zoo animals and captive non-equine ungulate herds. Pregnancy has been successfully 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).

In addition to monitoring of estrogen metabolites, pregnancy diagnosis via progesterone has been utilized, albeit to a somewhat lesser degree due to fluctuation in concentrations. A study measuring progesterone from the feces of captive giraffes at Busch Gardens found that concentrations in non-pregnant giraffes ranged significantly (3,420 ± 5290 ng/ml) in 41 samples from seven individuals. Extreme variability existed not only between individuals, but within samples from the same individual taken only a few days apart (Dumonceaux 2006). Another study in captive pregnant Red Brocket deer found significant variability in fecal progesterone concentrations throughout gestation (Krepschi 1995). Additionally, a study measuring fecal progesterone in Grevy's zebra mares found that concentrations

were quite variable throughout gestation, declining to or near levels seen during the estrous cycle at 1-2-week intervals, then increasing again (Asa et al 2001).

Pregnancy Determination and Management in the Mare

Pregnancy in the domestic mare can be determined by blood draw as early as 35 to 42 days, when equine chorionic gonadotropin (eCG) reaches detectable levels prior to declining to undetectable levels at approximately 100 days (Brinsko et al 2011). As bleeding feral mares is not possible without significant restraint, other means of pregnancy diagnosis have been examined.

Aside from the obvious necessity to obtain samples for pregnancy analysis in a non-invasive manner, the end goals in feral horse management differ significantly from those in place for domestic horses. For example, most domestic horse owners have at least a rough idea as to when breeding occurred, as well as the ability to handle their horses should the need arise for samples to be obtained. Conversely, as many feral horse herds are unused to prolonged human interaction, handling is not a realistic option. Additionally, unless closely monitored, breeding dates may be completely unknown.

As many managers of feral herds are interested in either decreasing or maintaining current populations, reproductive management related to measurement of pregnancy hormones should focus on hormones that both increase early in gestation, but also definitively differentiate from non-pregnant hormone concentrations as early as possible in pregnancy. Knowing the pregnancy status of the mares within their herds allows managers to better design their course of management practices season to season.

Since the initiation of measuring steroid metabolites, research has been completed in domestic and feral mares regarding determination of pregnancy. Of these studies, the majority examine estrogen, while a few examine progesterone. Although measurable in both the blood and feces of the pregnant mare, progesterone concentrations have been found to vacillate throughout gestation, making it an

unreliable indicator of gestation. In their study of saddle domestic mares, Holtan and colleagues (1975) found that peripheral plasma progesterone concentrations fluctuated widely throughout gestation (Figure 4). Although progesterone significantly increased at approximately day 28, reaching maximum values on day 64, it began a precipitous decline at 120 days to levels less than those seen in post-partum mares (Holtan et al 1975). While progesterone did increase in the last 30 days of gestation, measured concentrations did not exceed those observed during the estrous cycle (Holtan et al 1975). Therefore, progesterone would not be suitable for use to distinguish pregnancy status after 120 days of gestation, and especially unsuitable if the gestation timing was completely unknown, as is often the case in feral mares. Another study examining fecal progesterone in Lippizzan, Trotter and Thoroughbred mares found that although progesterone levels began to increase in the last three months of gestation, maximal levels weren't reached until approximately a month prior to parturition (Schwarzenberger 1991). If sampling in a feral population, it would be challenging to obtain a single sample from which pregnancy could be definitively diagnosed, unless taken during the last month of gestation. As managers of feral herds need to know pregnancy status as early as possible, waiting until the last 30 days of an approximately 345 day gestational period is not ideal.

In the pregnant mare, the estrogens found in increasing concentrations are estrone, estrone sulfate, estradiol, equilin, and equilinen (Bamberg et al 1984). Although equilin and equilinen are found in both the blood and urine in measurable amounts, they are sterically very similar, making them difficult to differentiate between (Bhavnani 1988). Regarding the other estrogens, there have been several studies examining pregnancy status in domestic and feral mares; utilizing urine, feces or blood. While measurement in urine is possible in feral mares (Kirkpatrick et al 1988; Henderson et al 1999), it's unpractical and time-consuming, requiring extraction of urine from the soil.

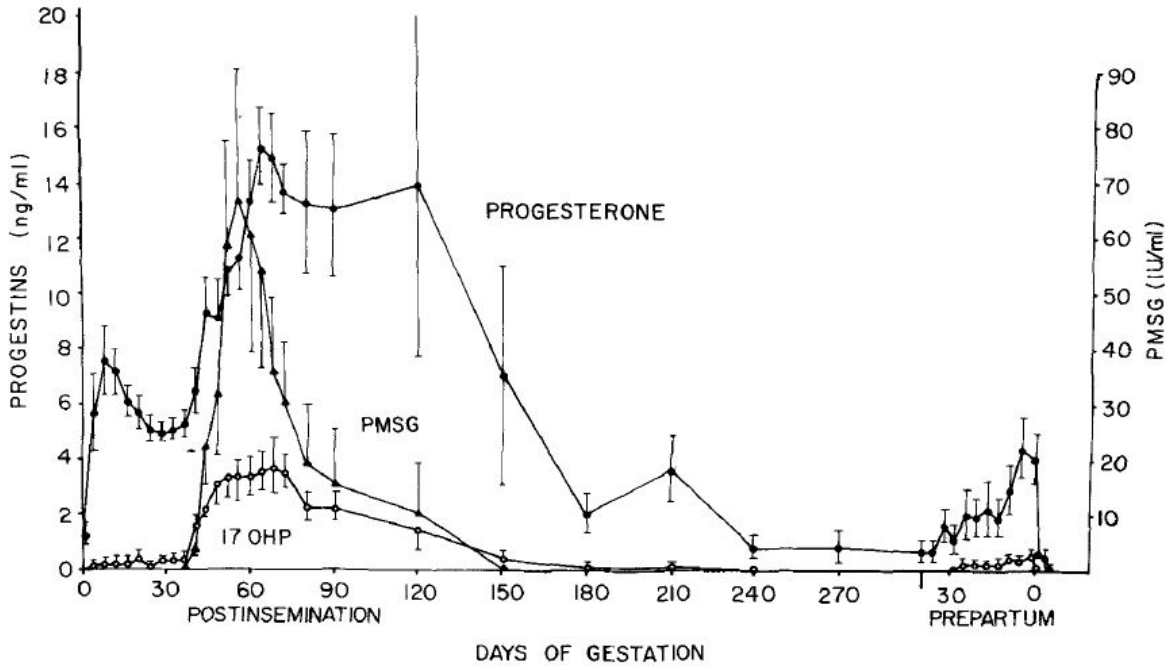


Figure 4. Plasma progesterone through gestation in the domestic mare (adapted from Holtan et al 1975)

Of the equine studies measuring fecal estrogens, the most widely measured metabolite has been estrone sulfate, which is also the most abundant in circulation (Raftogianis et al 2000). In an extensive study using 116 nonpregnant domestic mares and 39 pregnant mares, Henderson and colleagues found that of the pregnant mares sampled at least 150 days post-breeding, 93% of the collected fecal samples resulted in estrone sulfate concentrations of >80 ng/g. None of the fecal samples from non-pregnant mares returned values greater than 80 ng/g feces, with only five non-pregnant mare samples above 65 ng/g for fecal estrone sulfate (Henderson et al 1999). This study found that pregnant mares sampled at least 150 days post-mating and with fecal estrone sulfate values > 80 ng/g of fecal estrone sulfate were unequivocally pregnant (Henderson et al 1999). The following year, a similar study measuring estrone sulfate in feral mares found that while the first 100 days of gestation resulted in concentrations similar to nonpregnant mares, samples taken from mares at approximately 150 – 200 days of gestation resulted in values of >100 ng/g feces (Linklater et al 2000). Non-pregnant feral mare fecal estrone sulfate values in this study were consistently less than 57 ng/g of fecal estrone sulfate (Linklater et al 2000).

Fecal estrone sulfate is not the only estrogen that has been examined in the mare; a few studies have measured pregnancy status using total fecal unconjugated estrogens. During a feral mare study, Kirkpatrick and colleagues determined that fecal samples obtained between days 120 – 180 post-breeding resulted in mean values of 3.18 ± 0.70 ng/g of total unconjugated fecal estrogens in pregnant mares, compared to 0.552 ± 0.08 ng/g of total unconjugated fecal estrogens in non-pregnant mares (Kirkpatrick et al 1989). These results were markedly different from those found by Bamberg, in which pregnant mare concentrations ranged between 100 – 300 ng/g of total unconjugated fecal estrogens if sampled at least 120 days after breeding (Bamberg et al 1984). The mean values for nonpregnant mares in this study were 4.1± 3.4 ng/g of total unconjugated estrogens (Bamberg et al 1984).

Although it is not feasible to measure estrogens in the blood of feral mares, it is in domestic mares. Researchers measured plasma concentrations of E_1 (estrone, equilin, equilinen) and E_2 (estradiol 17β and estradiol 17α). Concentrations of E_1 significantly increased 90 days post-insemination, with means of 43 pg/mL E_1, while E_2 concentrations significantly increased at approximately 150 days, with means of 34.6 pg/mL E_2 (Nett et al 1973). Both concentrations continued to rise early in gestation, with E_1 peaking at 210 days post-insemination, and E_2 at 240 days (Nett et al 1973). Both E_1 and E_2 dropped following their respective maximum values, decreasing to concentrations seen in post-partum mares by the end of gestation (Figure 5) (Nett et al 1973).

While there have been no fecal estradiol studies specific to the equine, there has been one completed in the Grevy's Zebra (*Equus grevyi*) (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 into the 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). The study used 3 zebra mares, with a mean gestational length of 372 days. Fecal estradiol levels began to rise at a mean of 88 days into gestation; significantly increasing at approximately day 120 over cyclic patterns (Asa et al 2001). Although this study was completed in equids with longer gestational periods than equine, fecal estradiol concentrations still increased significantly at a timepoint similar to increases seen in equine mares.

Radioimmunoassay

RIA was initially developed to measure the distribution and clearance of insulin in diabetic and non-diabetic individuals (Yarlow and Benson 1959). Following its initial use, RIA was primarily utilized for measurement of peptide hormones, but by the late 1960's was being applied to other disciplines, including toxicology, oncology, and infectious disease (Patrono and Peskar 1987).

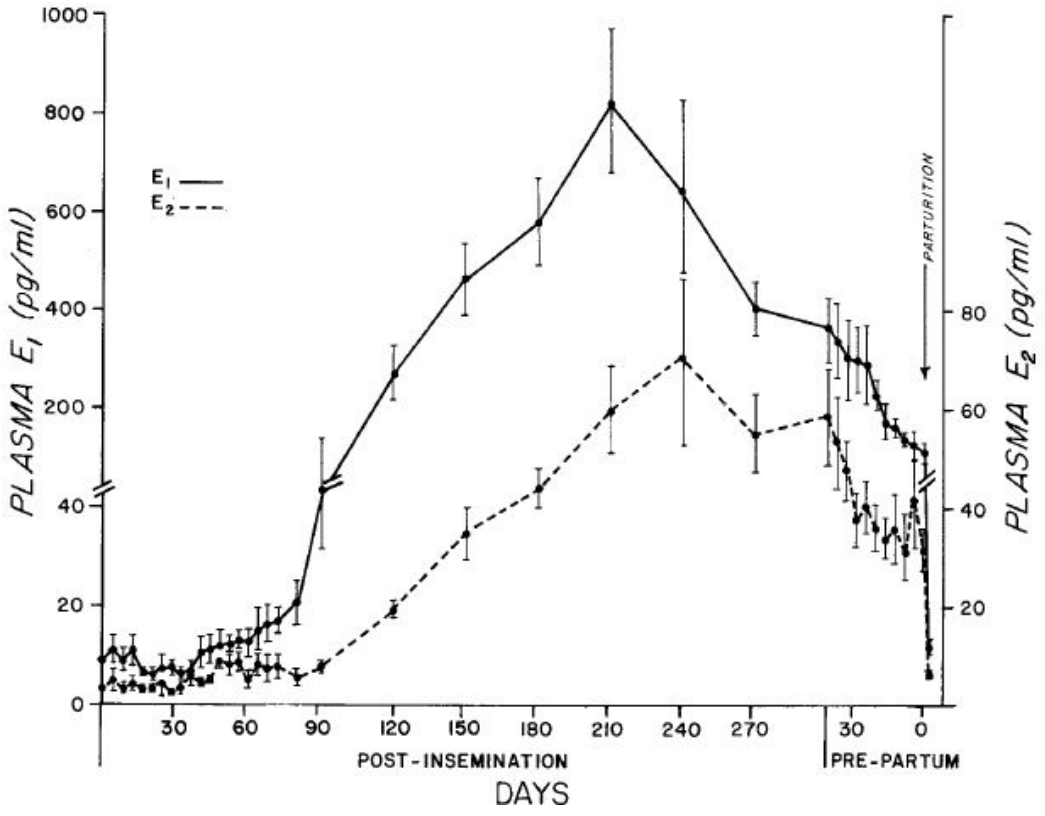


Figure 5. Plasma E_1 (estrone, equilin, equilinen) and E_2 (estradiol 17β and estradiol 17α) in pregnant mares throughout gestation (Nett et al 1973).

RIA is an immunochemical method for quantifying substances through competitive binding of ligands to antibodies of high affinity. Quantification is based on the ability of the endogenous (non-radioactive) ligand of a sample to compete in binding antibody against a radioactive form of the ligand (Nett and Malvey 1999). Prior to achieving quantification of non-radioactive ligand in a sample, the percentage of radioactive ligand bound to primary antibody, in the absence of competition, is measured. This is known as the B_0 (Nett and Malvey 1999). The B_0 is important because it dictates the basis for the calculation of the non-radioactive ligand, and B_0's with primary antibody dilutions that result in 30-50% of radioactive ligand bound to primary antibody are generally chosen, as illustrated in Figure 6 (Nett and Malvey 1999).

The percentage of antibody-bound radioactive ligand directly correlates to the ability for the non-radioactive ligand to competitively bind antibody when added to the mixture. Quantification of non-radioactive ligand is achieved through competitive binding with radioactive ligand for antibody binding sites, with both antibody dilution and radioactive ligand held constant. If a B_0 with primary antibody dilution is chosen that results in <50% binding of radioactive ligand to antibody, then more non-radioactive ligand will need to be added to ensure competitive binding and inhibition of the radioactive ligand (Nett and Malvey 1999). While less radioactive material bound to antibody results in assays with higher sensitivity, as more non-radioactive ligand is bound, there still needs to be sufficient bound radioactive ligand for the assay to be accurate. An assay that does not contain enough radioactive material results in fewer counts, more error, and more variability.

As quantification of RIA is achieved through measurement of ligand bound to antibody, it is imperative that the antibody used is specific to the ligand of interest. To produce ligand-specific antisera, the ligand needs to be capable of eliciting an immune response, the essential features of which include size larger than 3000 Daltons, rigidness, chemical complexity, and foreignness (Tizard 1982).

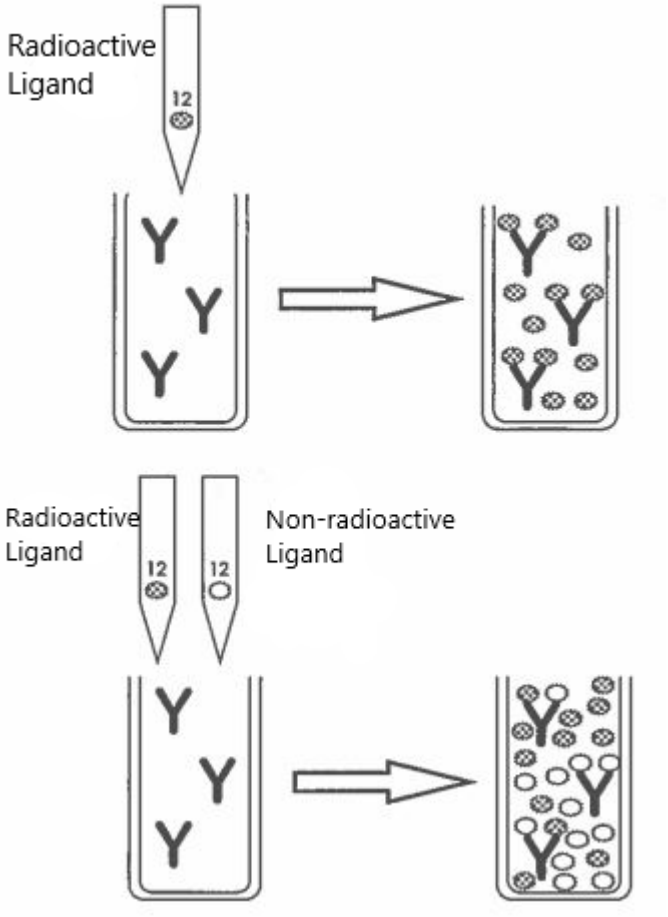


Figure 6. Antibody and radioactive ligand are initially combined in concentrations such that approximately 50% of the radioactive ligand is bound to antibody. If nonradioactive ligand is added to the mixture, and radioactive ligand and antibody concentrations held constant, the resulting binding of radioactive ligand to antibody is reduced due to competitive binding by the non-radioactive ligand (Nett and Malvey 1999).

Some ligands do not meet that criteria on their own, so must first be altered via attachment to a larger molecule, or carrier, such as bovine serum albumin (BSA), after which they are capable of antibody production, making them antigenic (Tizard 1982; Nett and Malvey 1999). Once attached to a carrier molecule, ligands are referred to as haptens. When conjugated to carriers, haptens can be mixed with adjuvant to further stimulate the immune response and injected into a host. While rabbits are often used as hosts due to both their handleable size and ability to produce relatively large amounts of antiserum with limited amounts of ligand used, other commonly used animals include sheep and goats (Nett and Malvey 1999; Leenaars and Hendriksen 2005). Immunogenic injections into a host causes the immune system to produce antibodies, or immunoglobulins, specific to the injection, over the course of several weeks (Nett and Malvey 1999). The time course of the immunogenic response varies, although administration of a booster injection timed with decreasing systemic blood antibody concentration can result in an extreme response of increased antibody titers (Nett and Malvey 1999).

The quantitative ability of an RIA to successfully measure ligands of biological samples is based assay sensitivity, which is defined as the lowest concentration of non-radioactive ligand that the assay is able to measure (Midgley et al 1969). The sensitivity of an RIA is indicated by the point of interception on the x-axis by a 95% confidence interval calculated from the B_0 (Nett and Malvey 1999).

RIA sensitivity is also linked to the type of radioisotope used, and ^{125}iodine (^{125}I) is a common choice. It can interact with and attach to a hapten linked to tyrosine methyl ester (TME), a derivative of tyrosine (Nett and Malvey 1999). Although previous equine studies have utilized ^{3}H estrogens to measure fecal estrogens (Bamberg et al 1984; Mostl et al 1984), there is a compelling reason to use ^{125}I estrogens. Radioiodinated haptens have a much higher specific activity than ^{3}H haptens and undergo disintegrations 75 times more rapidly than ^{3}H, resulting in the necessity of 75 times as many ^{3}H molecules to generate the same levels of radioactivity as ^{125}I (Nett and Malvey 1999). Therefore, significantly less mass of radioiodinated haptens are required to release a defined amount of

radioactivity, as compared to ^{3}H hapten mass (Nett and Malvey 1999). Additionally, as the sensitivity of a RIA is relative to the measurable amount of radioactive disintegrations from radiolabeled ligand bound to antibody, using ^{125}I labeled haptens results in assays two or three orders of magnitude more sensitive than those using ^{3}H labeled haptens (Nett and Malvey 1999).

To quantify ligand bound to antibody, the radioactive form of a ligand is used, with the resulting specificity of the assay directly correlated to the purity of the radioactive ligand (Nett and Malvey 1999). This necessity is related to the antiserum produced by the host. While it contains the antibodies it was produced for, it also retains others relative to any other foreign substance to which the host had been previously exposed, which can lead to cross-reactivity of the antiserum to different analytes that are structurally similar. If a nonpure radioactive ligand contains proteins to which the host has antibodies, they will bind, resulting in interference of quantification to the ligand of interest (Nett and Malvey 1999). To ensure both the purity of the radioactive ligand, as well the RIA specificity, it is crucial to confirm that other components within the measured sample do not interfere with the radioactive ligand, and is generally considered to be the most important indicator in assay reliability. If a radioactive ligand is pure, anything that inhibits its binding with antibody is most likely to either be an identical or closely related substance to the ligand (Nett and Malvey 1999).

In their study examining plasma estrogens in domestic mares, Nett and colleagues developed an RIA with specificity and sensitivity to estrone and estradiol 17β (Nett et al 1973). They utilized column chromatography to separate the estrogens, with one fraction containing estrone, equilin, and equilinen (E_1 fraction), and the other containing estradiol (E_2 fraction) (Nett et al 1973). They determined that the antiserum used for the RIA did not cross-react with non-estrogenic steroids, and had limited cross reactivity with other estrogen metabolites, which were differentiated via inhibition curves (Nett et al 1973). Inhibition curves compare a standard of a substance of interest to other similar samples, and parallelism of all curves is indicative that substances other than the one of interest are not causing

antibody binding interference, and are not interacting with the antiserum used (Nett and Malvey 1999). Pooled E_1 (estrone-equilin-equilinen) and E_2 (estradiol 17β and α) fractions were parallel to those obtained with estrone and estradiol 17β standards (Nett et al 1973), indicating a lack of binding inhibition, and an RIA with high specificity. These assays also resulted in sensitivities of 4 pg/tube for both estrone and estradiol 17β (Nett et al 1973).

The last points of interest to be considered for reliable RIA are precision and accuracy. Precision is the amount of variation noted in the estimated non-radioactive ligand (Midgley et al 1969). While variances within an assay are affected by pipetting error relative to assay preparation, another effect is the counting error produced by the gamma counter used. This error is calculated by taking the square root of the total radioactive counts, divided by the total counts (Nett and Malvey 1999). The counting error can then be used to establish estimates of variation both within an assay, and between two identical assays, cumulatively known as coefficients of variation, which should be less than 20% (Nett and Malvey 1999). Assay accuracy can be defined as the mean of an infinite number of measurements of a material, and how they align with the exact amount of the material present in a sample (Midgley et al 1969). An optimal method for examining RIA accuracy is to compare results obtained from several RIAs with a variety of preparations, such as similar antibodies (Midgley et al 1969). If relative assay results agree, it is likely that the same substance is being measured across assays. Another option is through weighing of a known amount of the ligand, and then adding varying amounts to a biological fluid of the same species that is lacking the ligand; for example, adding varying concentrations of estradiol to the serum of an ovariectomized mare. The concentration of ligand in the serum is then determined by RIA, and correlation graphed between ligand added (x-axis) and amount measured (y-axis). If the result is a line with a slope of 1, the two amounts are equal (Nett and Malvey 1999).

The final consideration to be made in RIA is how best to separate free ligand from bound, so that assay quantification can be completed. While there are a variety of techniques, the most common

method utilized is termed the ‘double-antibody procedure’, and requires that of a second antibody, generated against immunoglobulins specific to the species in which the primary antibody was produced (Nett and Malvey 1999). Secondary antibody is added to the mixture after the reaction of primary antibody and ligand has completed. The secondary antibodies then bind to the primary antibody and ligand, causing immunoprecipitation of the ligand/antibody complex from the solution. (Nett and Malvey 1999).

CHAPTER 2

Measurement of Fecal and Serum Estradiol in the Domestic Mare

Introduction

Measurement of estrogen and estrogen metabolites for pregnancy determination was originally developed in zoos as a means of accurate pregnancy diagnosis without the physical stressors of restraint (Lasley et al 1991). Since their initial use, they have also been utilized in non-exotic species, including both domestic and feral mares. There have been numerous equine specific studies that have examined fecal estrogen and metabolites as a means of pregnancy diagnosis, as well as definitive timing for optimal sample collection. Those examining total fecal unconjugated estrogens have determined pregnancy from samples collected between 120-180 days (Bamberg et al 1984; Kirkpatrick et al 1989), while those measuring fecal estrone sulfate report fecal concentrations relative to definitive diagnosis from samples taken at 150 days of gestation and beyond (Henderson et al 1998 and 1999; Linklater et al 2000). While there have not yet been any equine studies focusing on fecal estradiol measurement, plasma estradiol concentrations of pregnant mares were found to differentiate from cycling mares at approximately 150 days of gestation (Nett et al 1973).

This study was designed to examine changes in fecal estradiol concentration throughout gestation, using serum estradiol levels as a comparison tool. The objectives of this study were: 1) to determine a reliable cut-off concentration that clearly delineates 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 surpass 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. Table 1 depicts the demographics specific to the cycling mares. 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, as shown in the second portion of Table 1.

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.

Table 1: Domestic mare sampling demographics including age and breed of the non-pregnant cycling mares and pregnant mares, as well as the day of pregnancy the first sample was taken in pregnant mares, and the day of parturition. Asterisks indicate samples taken the day of parturition.

CYCLING MARES

Mare	Age(years)	Breed
00	11	Grade
24	15	Grade
49	9	Grade
95	10	Grade
124	10	Paint
150	11	Grade
231	11	Grade
443	10	Grade

GESTATIONAL MARES

Mare	Age (years)	Breed	First Sample	Parturition*
15029	9	Grade	d27	d356*
15024	8	Grade	d28	d331
15105	6	Grade	d53	d355*
15099	10	Grade	d54	d351*
15042	13	Grade	d59	d340*
15110	8	Grade	d88	d350*
15004	7	Grade	d89	d342
15049	16	Grade	d119	d361

* sample taken that day

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 lyopholization. The dishes were then frozen at -20°C for 30 minutes prior to placement into a lyopholizer 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 (DDH_2O). 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 µL equine sample aliquot in a 16x150 mm tube, two 16x150 mm tubes were labeled with the same sample number, each containing 500 µL of DDH_2O for washing. Each 250 µL QC aliquot also had the same number of tubes prepared. For the second extraction, 5 mL of diethyl ether was added to each 500 µL equine aliquot or 250 µL 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 µL DDH_2O 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 µL for the mare samples, and 250µL 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 µL 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 µL for equine samples, and 250 µL 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 17β 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 µL of extracted sample was added to each tube, after day 100, 10 µL of extracted sample was used. Extracted serum samples collected from pregnant mares were divided at gestation day 130; prior to day 130, 200 µL of sample was added to each tube, after day 130, 50µl was added. For cycling mares, 200 µL 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 µL (Nett 2005). Five hundred microliters of buffer was

added to NSB tubes and BC tubes. One hundred microliters of iodinated estradiol 17β-6-TME was added to all tubes in the assay, followed by 200µL 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 µL 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 17β, 12% with estrone, 7% with estradiol 17α, 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 17β 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.

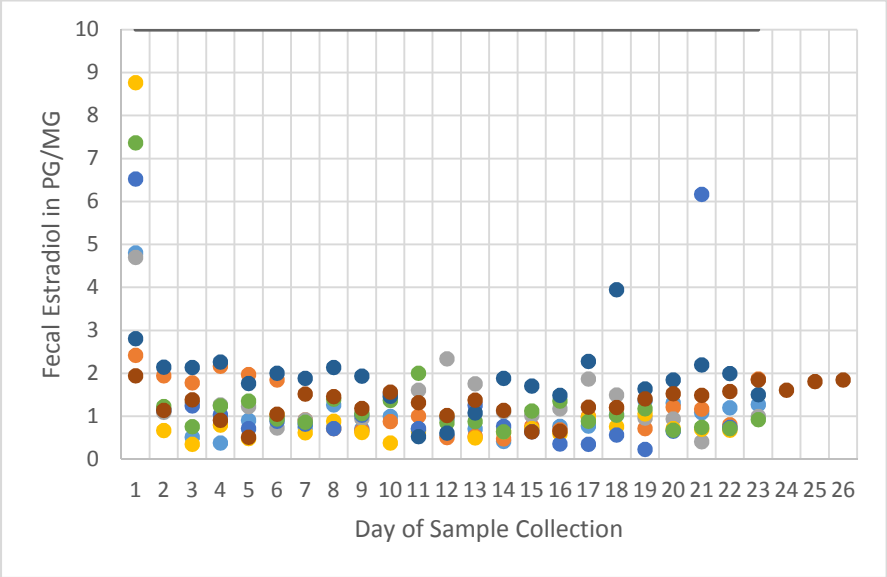
Of the 185 fecal samples collected from cycling mares, none of them were above the calculated COC, which is depicted in Figure 7.

Serum concentration of estradiol 17β 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 8.

Gestational Mares

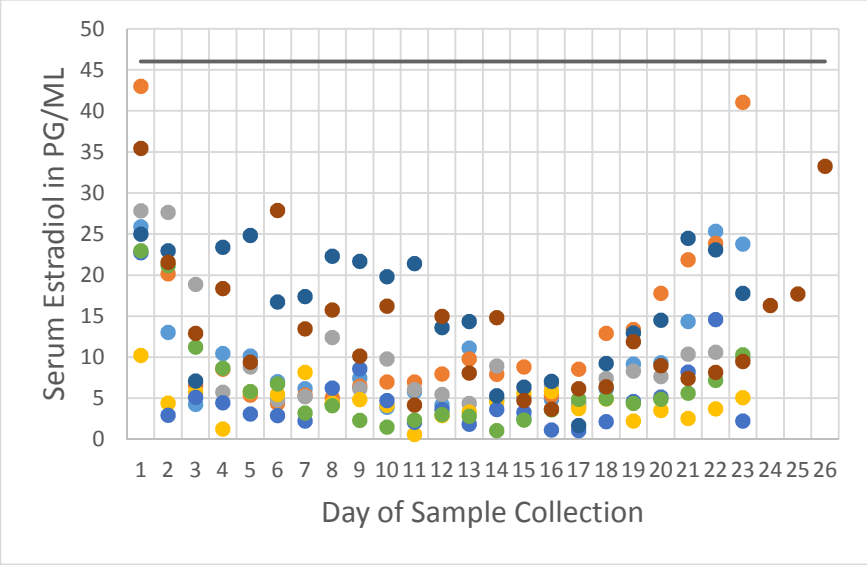
The average gestational length for mares in this study was 348 days. The range in concentrations of fecal estradiol 17β in eight gestational mares was 1 - 134 pg/mg of feces. Concentrations increased early in pregnancy as pregnancy progressed, surpassing the calculated COC of 10 pg/mg feces at an average of 105 days of gestation, before decreasing below the COC directly before parturition, as indicated in both Figure 9A and Table 2.

Of the 310 fecal samples, 43 were collected prior to the cut-off day (COD) of 105 days of gestation. Of those 43, 34 failed to surpass the COC of 10 pg/mg feces, while nine did. Of the remaining 267 samples taken past the COD, all but 9 exceeded the COC. Three of those nine were from samples taken close to the COD, which were day 108 (d108) for mare 15105, d111 for 15110, and d105 for 15099. The remaining 6 were taken close to the day of parturition, with 4 taken the day of parturition for mares 15042, 15029, 15105, and 15110, and two taken from the day prior from mares 15024 and 15105. Figure 9B illustrates the fecal concentrations of the 8 gestational mares 30 days pre-parturition, of which the 6 values that dropped below the COC at the end of gestation can easily be seen.



Cycling Mares	Highest Fecal Concentration (pg/mg)
00	5
24	2
49	5
95	9
124	7
150	7
231	4
443	2
AVG	5
SD	2
Avg +2SD (Cut-Off)	10

Figure 7: 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.



Cycling Mares	Highest Serum Concentration (pg/mL)
00	26
24	43
49	28
95	10
124	23
150	23
231	25
443	35
AVG	27
SD	10
Avg+2SD (Cut-Off)	46

Figure 8. 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.

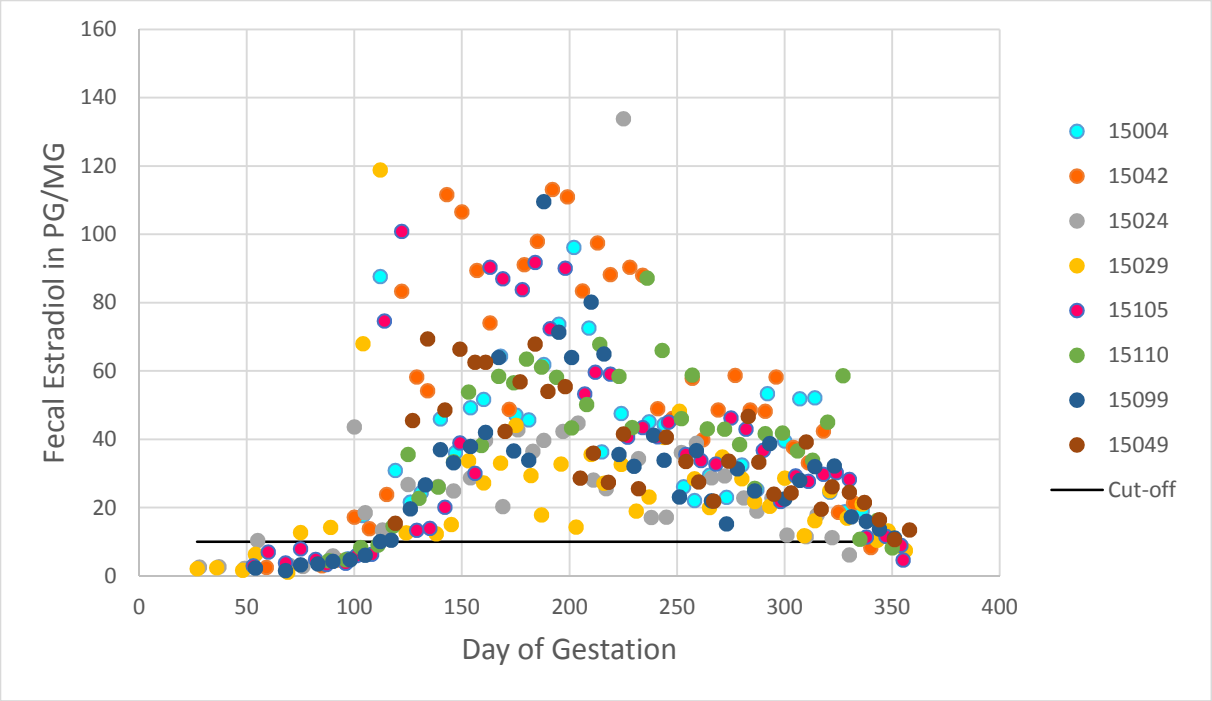


Figure 9A. Weekly fecal estradiol 17β concentrations in pg/mg of feces throughout gestation in 8 domestic mares. Cut-off value is 10 pg/mg of feces and is indicated by black horizontal line.

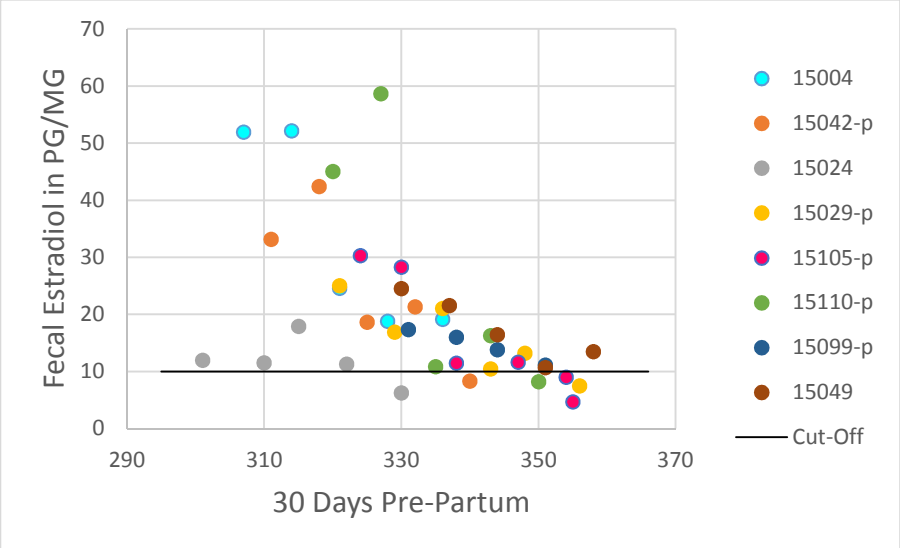


Figure 9B. Fecal estradiol 17β concentrations 30 days prior to parturition in 8 domestic mares. Cut-off value is 10 pg/mg of feces and is indicated by the black line. Mare numbers labeled with a -p indicate samples taken on the day of parturition.

Table 2. The first day of gestation for each of 8 pregnant domestic mares that surpass the calculated cut-off values for feces and serum.

Gestational Mares	Day Surpassed Avg+2SD using highest fecal value: 10 pg/mg	Day Surpassed Avg+2SD using highest serum value: 45.875 pg/mL
15004	104	140
15042	100	134
15024	100	113
15029	75	138
15105	114	129
15110	118	125
15099	112	117
15049	119	127
	Average: 105.25 = 105 days	Average: 127.88 = 128 days

Serum estradiol 17β 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 10A and Table 2). 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. Two of those 13 were taken at gestation days 131 (mare 15004) and 129 (mare 15042), both of which were relatively early in gestation. Mare 15004 had six mid gestation samples between days 154 and 321 that dropped below the COC, while the remaining 5 were from 4 mares. Two were from the same mare, 15042, with one eight days prior to parturition, and the other the day of parturition. The remaining three, from mares 15024, 15029 and 15110 were taken the day of parturition. Figure 10B illustrates the serum estradiol concentrations from the 8 gestational mares 30 days pre-partum.

Discussion

The results of this study indicated that measurement of estradiol 17β through the use of RIA 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.

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. This decision was contingent on a few factors.

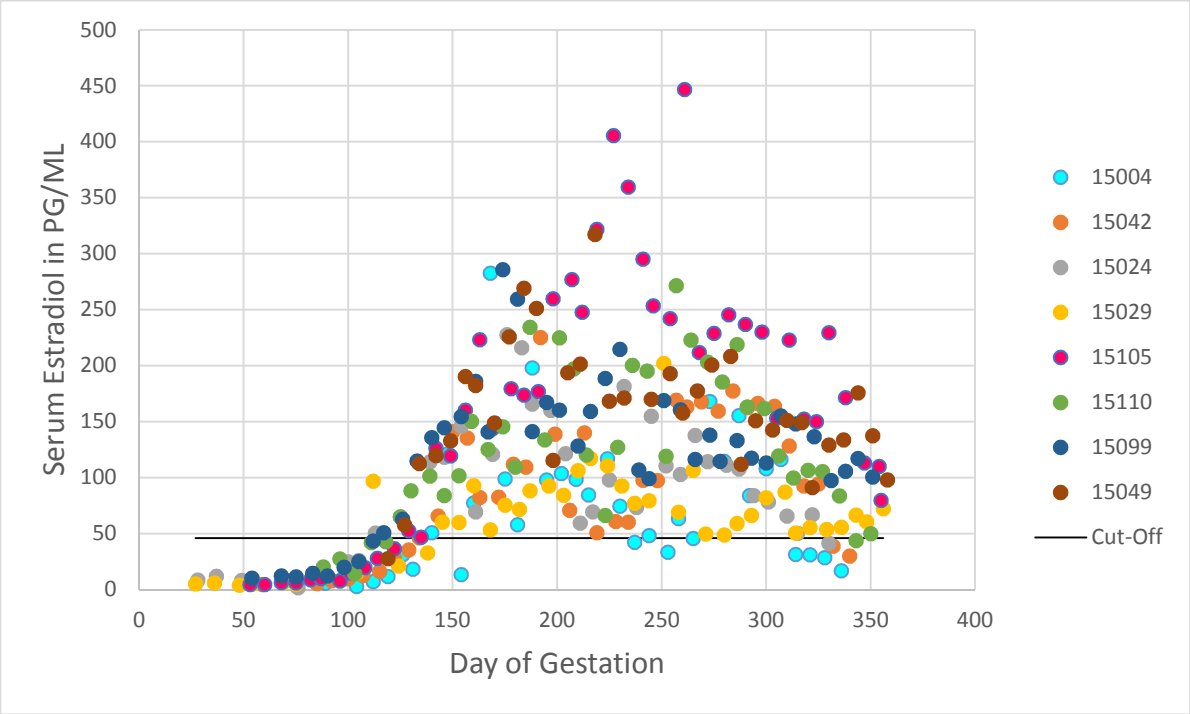


Figure 10A. Weekly serum estradiol 17β concentrations in pg/mL of serum throughout gestation in 8 domestic mares. The cut-off value is 46 pg/mL of serum and is indicated by black horizontal line.

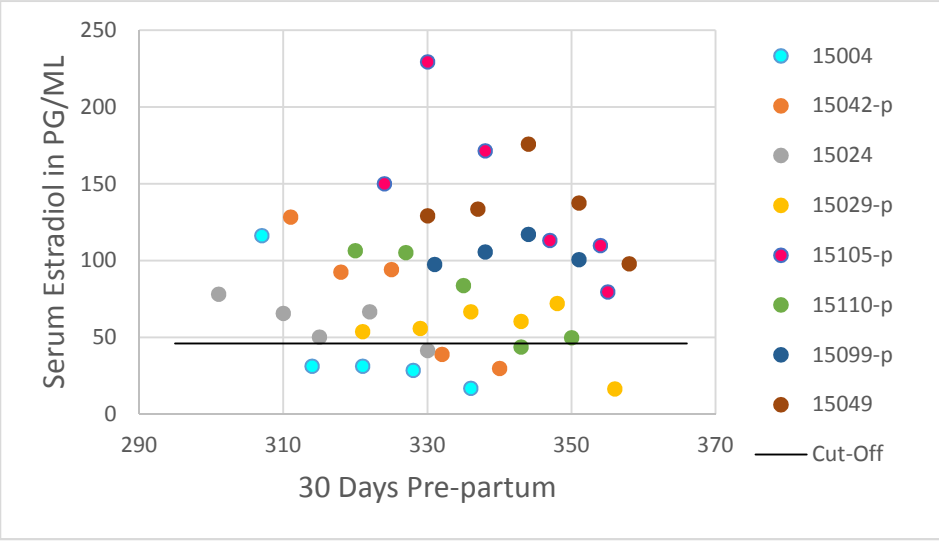


Figure 10B. Serum estradiol 17β concentrations 30 days prior to parturition in 8 domestic mares, with the 46 pg/mL cut-off indicated by black line. Mare numbers labeled with a -p indicate samples taken on the day of parturition.

The overall means of the non-pregnant mare values for both feces and serum were much lower than that of the mean from the highest concentrations (Table 3), resulting in COC of 2 pg/mg feces (mean +3SD), and 23 pg/mL serum (mean +3SD). These values were lower than some of the individual sample concentrations, and therefore not suitable for use in determination of pregnancy.

Although both of the calculated COC's using 3SD above the mean (12 pg/mg feces and 56 pg/mL serum; Table 3) surpassed all individual sample concentrations, they were quite conservative, having been calculated from the average of the highest individual concentrations of feces and serum from each mare. This would mean that determination of pregnancy relative to COD would be later in gestation, which wouldn't be ideal for feral horse managers, whose needs include the earliest possible detection of pregnancy for population management decisions. Therefore, calculations were made utilizing 2SD above the mean of the highest concentrations, which resulted in the COC's of 10 pg/mg of feces and 46 pg/mL of serum. While these concentrations surpassed all of the individual mare samples, they also had the advantage of earlier pregnancy detection and COD, compared to the more conservative calculations.

Of the 310 fecal samples collected from pregnant mares, 267 of them were obtained past COD 105. Of those 267, 258 surpassed the COC (96.6%), while nine of them did not (3.4%). Of the 9, three were near the COD: d108 (mare 15105), d111 (mare 15110) and d105 (mare 15099). While the following samples collected for each of these mares resulted in the definitive day of pregnancy determination based on the COC, a certain amount of variation is expected for timing of increases in fecal estradiol concentration. This is because the COD of 105 days was the average calculated from all of the individual mare values, as seen in Table 2. Additionally, from the 43 samples collected prior to COD 105, nine of them surpassed the COC, which again speaks to the calculation of the COD. Therefore, the failure of the 3 samples taken so closely after the COD to surpass the COC of 10 pg/mg of feces is not overly alarming.

Table 3. Calculation of cut-off values for feces and serum from domestic mares. Boxed values in the left panel were the ones chosen for use.

Cycling Mares	Highest Fecal Concentration	Cycling Mares	Average Fecal Concentration
00	5	00	1
24	2	24	1
49	5	49	1
95	9	95	1
124	7	124	1
150	7	150	1
231	4	231	2
443	2	443	1
AVG	5	AVG	1
SD	2	SD	0.3
Avg +3SD	12	Avg +3SD	2
Avg +2SD	**10**	Avg +2SD	2
Cycling Mares	Highest Serum Concentration	Cycling Mares	Average Serum Concentration
00	26	00	10
24	43	24	13
49	28	49	10
95	10	95	5
124	23	124	5
150	23	150	6
231	25	231	16
443	35	443	14
AVG	27	AVG	10
SD	10	SD	4
Avg+3SD	56	Avg +3SD	23
Avg+2SD	**46**	Avg +2SD	18

The remaining 6 samples taken after COD 105 that failed to surpass the COC were all from either the day of or directly prior to parturition. As it is known that plasma estradiol concentrations decrease during the last 30 days of gestation (Nett et al 1973), it would be expected that fecal estradiol concentrations would as well.

The resulting percentage of fecal samples that were over COC (96.6%) were similar to outcomes from previous studies examining fecal estrone sulfate, which resulted in 93% and 97.3% fecal concentrations above calculated cut-offs (Henderson et al 1998 and 1999).

Of the 317 serum samples taken from pregnant mares in the study, 252 were collected after the COD of 128 days, with 239 (94.8%) of them surpassing the COC of 46 pg/mL of serum. Of the 13 that failed to surpass the COC, two of them were near the COD: d131 (mare 15004) and d129 (mare 15042). The same rationale that was used for feces is applied here as well, which is that the COD was a calculated average determined from individual mares (Table 2), with some expected variation. Of the other 11 samples, six were from one mare, while five were taken from four mares roughly a week prior to parturition. As it is known that plasma estradiol decreases approximately 30 days prior to parturition (Nett et al 1973), the 5 samples with concentration decreases within eight days of parturition can be expected. As far as the six mid-gestational samples from mare 15004, that could just be individual variances, as no other sample resulted from fluctuations similar to hers.

Studies in the literature have reported definitive pregnancy determination through both fecal metabolite monitoring and measurement of plasma estradiol, with timing dependent on type of estrogen measured. When measuring total unconjugated fecal estrogens, pregnancy determination was made between approximately 120 and 180 days (Bamberg et al 1984; Kirkpatrick et al 1989), while fecal estrone sulfate concentrations resulted in measurable pregnancy status when samples were taken at least 150 days into gestation (Henderson et al 1998 and 1999). In the measurement of plasma estradiol,

concentrations relative to pregnant mares surpassed those of non-pregnant mares at approximately 150 days post insemination (Nett et al 1973).

In this study, fecal estradiol 17β concentrations of pregnant mares diverged from those of non-pregnant at an average of 105 days of gestation, while that of serum estradiol 17β diverged at a mean of 128 days. In the calculation for feces, one mare, 15049, was initially sampled on day 119 of gestation, which was also the first day that her fecal estradiol concentration surpassed the cut-off value (Table 2). When she is removed from the calculation for fecal estradiol, the average day for definitive pregnancy diagnosis drops to 103 days. In this instance, serum was unaffected, as the first concentration that surpassed the cut-off value was on day 127 of gestation. Using either average calculation for feces, the average day 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). As would be expected, the serum estradiol concentrations for pregnancy determination are similar to that of plasma estradiol (Nett et al 1973). Between the fecal and serum estradiol results, the fecal estradiol information is more crucial regarding feral horse management. The earlier that managers can confirm or discount pregnancy within their herds, the more time they have for management decisions.

For this method to be the most effective, while it would be ideal if managers could collect a few fecal samples throughout the breeding season, the most important sample would be one collected at least 3.5 months after the conclusion of the breeding season. This would ensure that any mares bred at the end of the season would have progressed far enough into gestation for concentrations of fecal estradiol 17β to be discernable from those of non-pregnant mares. Additionally, at that point, any concentrations returned below the cut-off could be concluded as not pregnant, as mares are not capable of ovulation during anestrus.

CHAPTER 3

Measurement of Fecal and Serum Estradiol in the Feral Mare

Introduction

While there are several feral horse populations throughout the United States (BLM 2020; NPS 2006), this study examined fecal and serum samples from feral horses in Theodore Roosevelt National Park (THRO). The 47,000-acre fenced park is located in Medora, North Dakota, and currently houses approximately 167 feral horses, as well as several hundred bison, antelope, and smaller mammals (THRO 2020). Historically, the feral horse herd at THRO was extensively managed through large roundups, but the park presently oversees equine numbers through fertility control and small gathers. Therefore, knowing pregnancy status of individual mares would be helpful when making decisions about contraceptive administration, and which horses to remove from the park.

The feral mares in this research are part of a GonaCon-Equine study in THRO. GonaCon-Equine is an immunocontraceptive approved for use in adult horses and burros by the Environmental Protection Agency (EPA) as a restricted use pesticide (Baker et al 2018). The formulation of GonaCon-Equine utilizes a non-biodegradable oil in water-based emulsion that contains immunostimulatory killed mycobacteria (Baker et al 2018). When injected into the muscle of the recipient animal, a slow release depot forms, which produces prolonged efficacy effects (Baker et al 2018).

As described in Chapter 2, the measurement of fecal and serum estradiol 17β concentrations in the mare is a reliable means of determining pregnancy status. After 105 days of gestation and until directly before parturition, fecal estradiol concentrations above 10 pg/mg are a reliable indicator of pregnancy, while after 128 days of gestation and approximately a week prior to parturition, serum estradiol concentrations above 46 pg/mL serum are indicative of pregnancy. Therefore, 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.

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

The method used for extraction of domestic mare samples was also utilized in this study. The extraction methods below have been taken from the description in Chapter 2. Although extraction procedures differ for feces and serum, both methods utilize quality controls (QCs), which are 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 lyopholization. The dishes were then frozen at -20°C for 30 minutes prior to placement into a lyopholizer 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 (DDH_2O). 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 µL equine sample aliquot in a 16x150 mm tube, two 16x150 mm tubes were labeled with the same sample number, each containing 500 µL of DDH_2O for washing. Each 250 µL QC aliquot also had the same number of tubes prepared. For the second extraction, 5 mL of diethyl ether was added to each 500 µL equine aliquot or 250 µL 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 µL DDH_2O 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, although using the second set of washing tubes during the wash phase (Nett 2005).

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 µL for the mare samples, and 250µL 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 µL 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 µL for equine samples, and 250 µL 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).

Assay

An RIA specific for estradiol 17β 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 µL of extracted sample was added to each tube, after day 100, 10 µL of extracted sample was used. Extracted serum samples collected from pregnant mares were divided at gestation day 130; prior to day 130, 200 µL of sample was added to each tube, after day 130, 50µl was added. For cycling mares, 200 µL 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 µL (Nett 2005). Five hundred microliters of buffer was added to NSB tubes and BC tubes. One hundred microliters of iodinated estradiol 17β-6-TME was added to all tubes in the assay, followed by 200µL 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 µL 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 17β, 12% with estrone, 7% with estradiol 17α, 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

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. Figure 11A and 11B depict the fecal estradiol concentrations of pregnant mares for both roundups, for a total of 77 fecal samples. Figure 11A 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 12A and 12B show the serum estradiol concentrations of pregnant mares for both roundups, again for a total of 77 serum samples. Figure 12A illustrates the entire sample set for all mares, while Figure 12B 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.

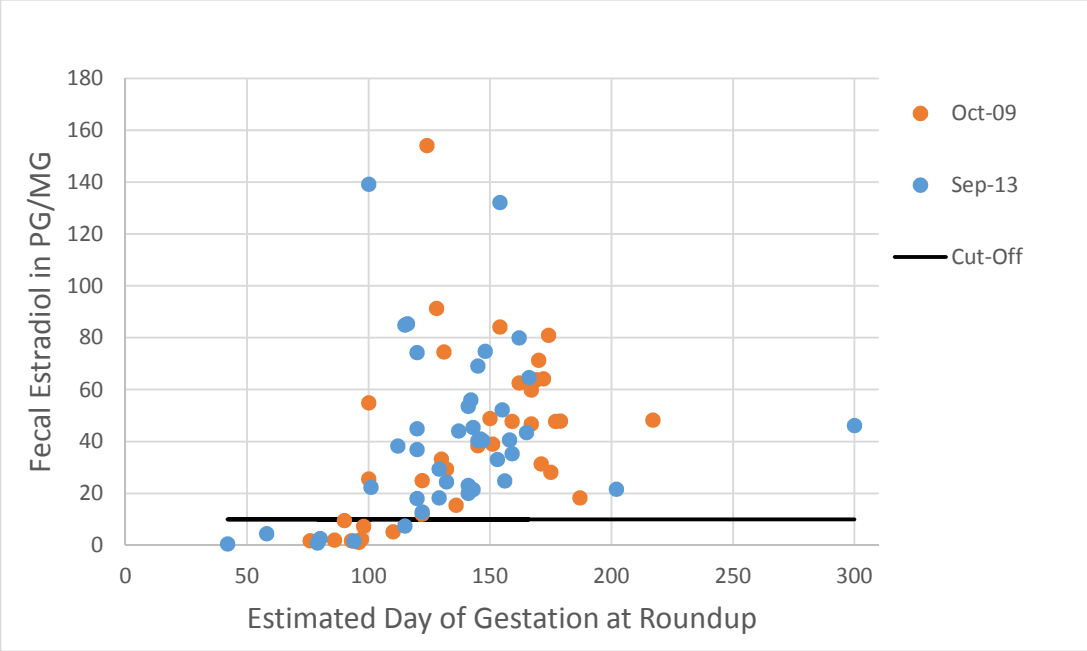


Figure 11A. Fecal estradiol 17β concentration from pregnant feral mare samples collected during the 2009 and 2013 roundups. The horizontal line indicates the cut-off concentration of 10 pg/mg feces.

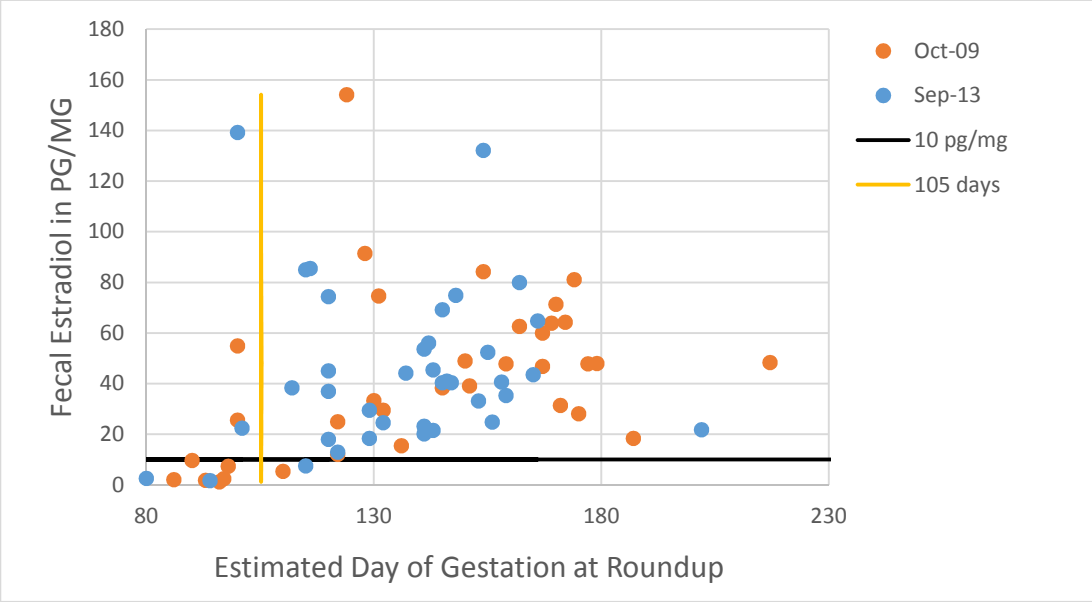


Figure 11B. Fecal estradiol 17β concentrations from pregnant feral mare samples collected after day 80 during the 2009 and 2013 roundups. The horizontal line indicates the 10 pg/mg cut-off concentration, while the vertical line indicates the cut-off day of 105.

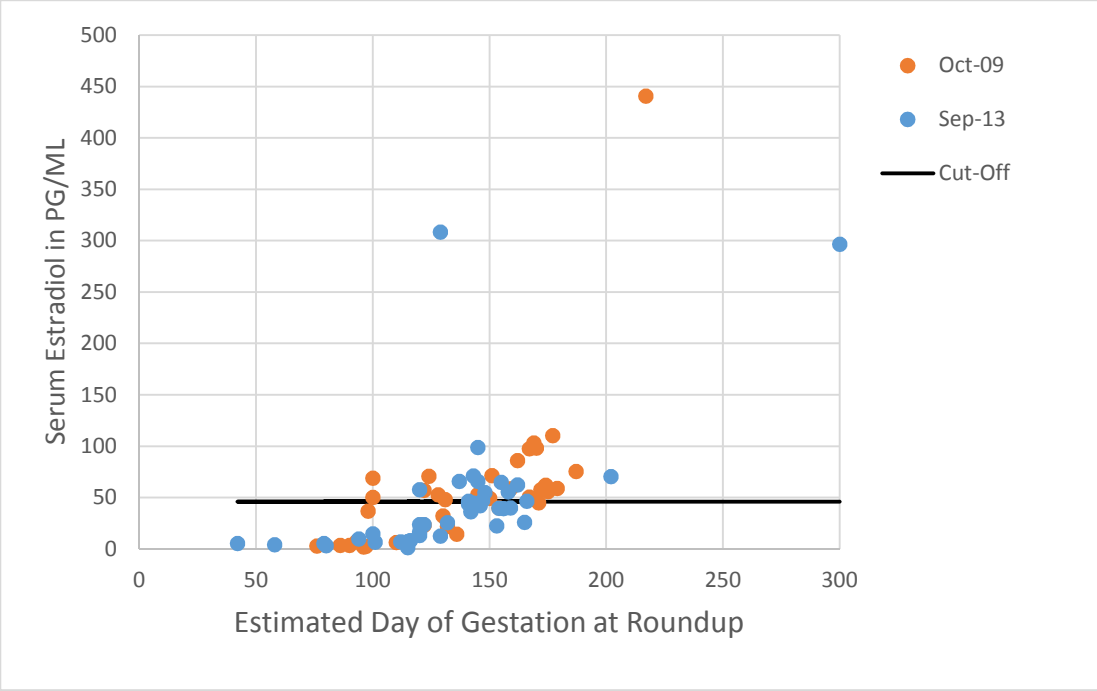


Figure 12A. Serum estradiol 17β concentration from pregnant feral mare samples collected during the 2009 and 2013 roundups. The horizontal line indicates the cut-off concentration of 46 pg/mL serum.

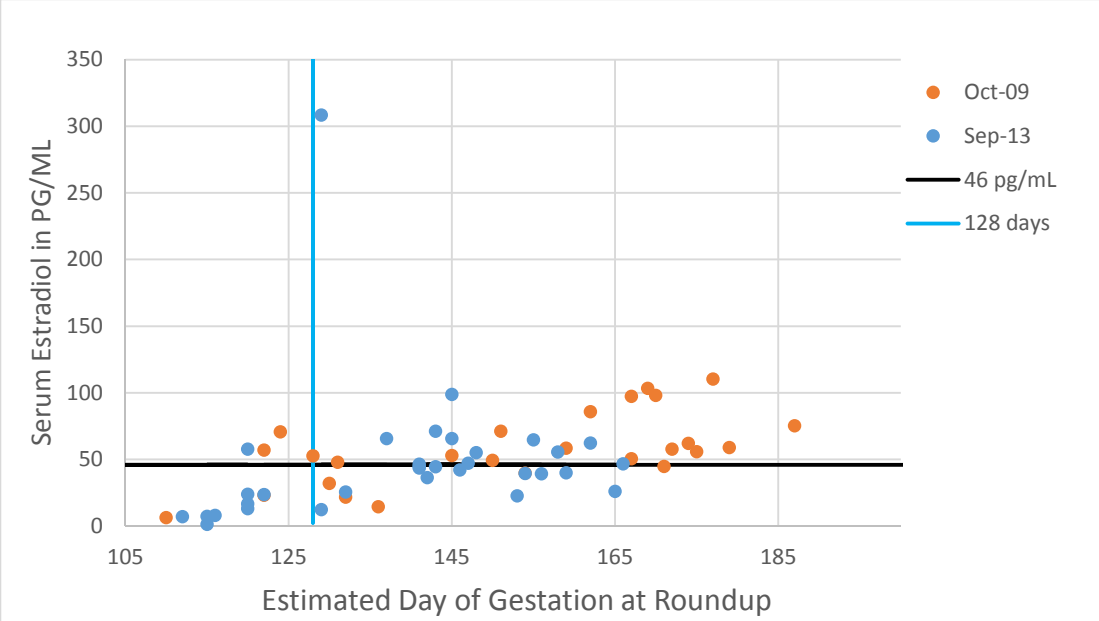


Figure 12B. Serum estradiol 17β concentrations from pregnant feral mare samples collected after day 105 during the 2009 and 2013 roundups. The horizontal line indicates the 46 pg/mL cut-off concentration, while the vertical line indicates the cut-off day of 128.

As shown in Table 4, of the 77 counted fecal samples collected between the two roundups, 62 surpassed the 105 day cut off, with 60/62 surpassing the COC of 10 pg/mg feces. The two samples that fell short of the COC are shown in Table 5. Of the 15 samples collected prior to day 105, 13 of them were below the COC of 10 pg/mg of feces. Regarding collected serum samples, 49 of the samples were collected past the serum COD of 128 days, with 34 of those samples surpassing the COC of 46 pg/mL serum. The collection day and corresponding concentration data for the 15 serum samples that surpassed the COD of 128, but not the COC is shown in Table 5. Of the 28 samples collected prior to day 128, 26 of them failed to surpass the COC, which is shown in Table 4.

Regarding non-pregnant mare data, there were a total of 19 mares during both roundups that were not pregnant. Table 6 displays both the fecal and serum concentrations obtained from these mares, none of which surpass the designated cut-offs. The samples in red indicate those from treatment 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 13A-B, 14 and 15A-D show samples collected during both roundups, as well as November – February across 6 years of sampling. Figure 13A indicates the 121 samples from non-pregnant mares throughout the study, none of which surpass the COC of 10 pg/mg feces. Figure 13B 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 14 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 14, Figures 15A-D display a series of graphs divided into gestation timepoints: 15A: 0-100 days of gestation ,15B: 101 – 151 days, 15C: 152 – 202, and 15D: 203 – parturition. Figure 15A 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 15B (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, 15C and 15D surpassed both COD as well as COC.

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 7, while Figure 16 illustrates the data from the remaining 19.

Table 4. 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 Pregnant: 36 Non-pregnant: 9	Mares: 51 total Pregnant: 41 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 5. 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 6. 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

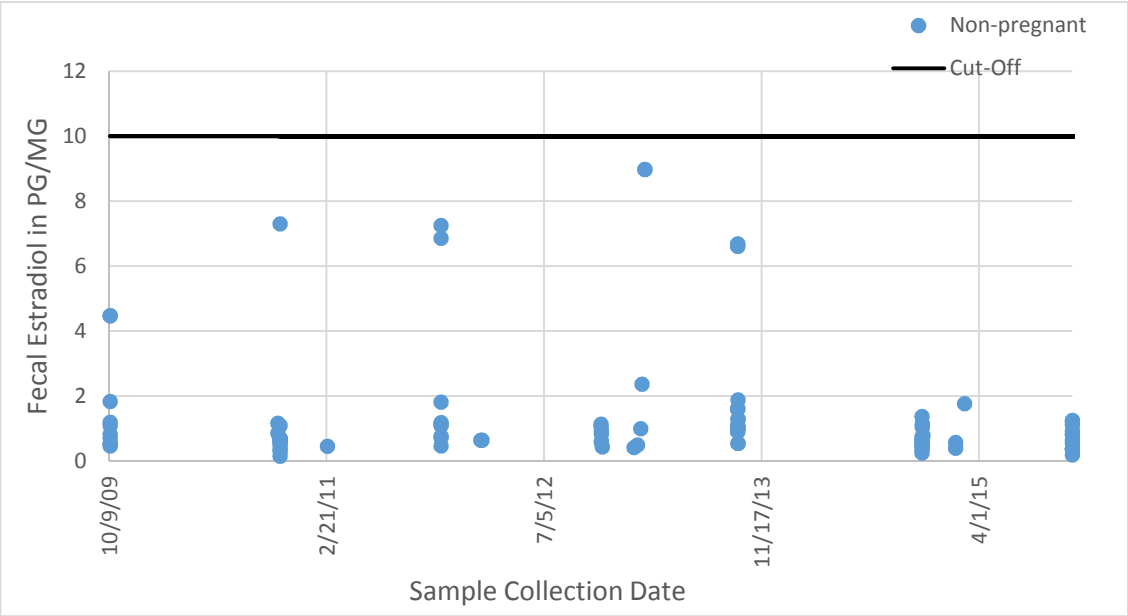


Figure 13A. Total distribution of fecal estradiol concentrations in pg/mg from collected samples of non-pregnant feral mares. The horizontal line indicates the cut-off concentration of 10 pg/mg feces.

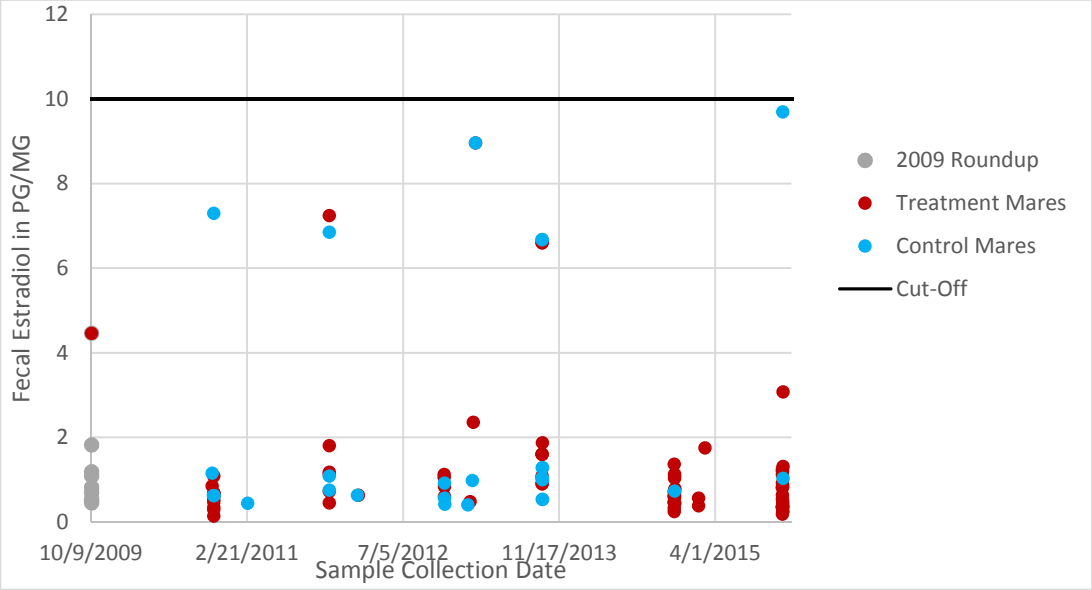


Figure 13B. Fecal estradiol concentrations in pg/mg from collected samples of non-pregnant feral mares. Samples are delineated as those collected at the 2009 roundup, or start of the study, as well as during proceeding years, labeled as treatment and control mare samples. The horizontal line represents the 10 pg/mg cut-off concentration.

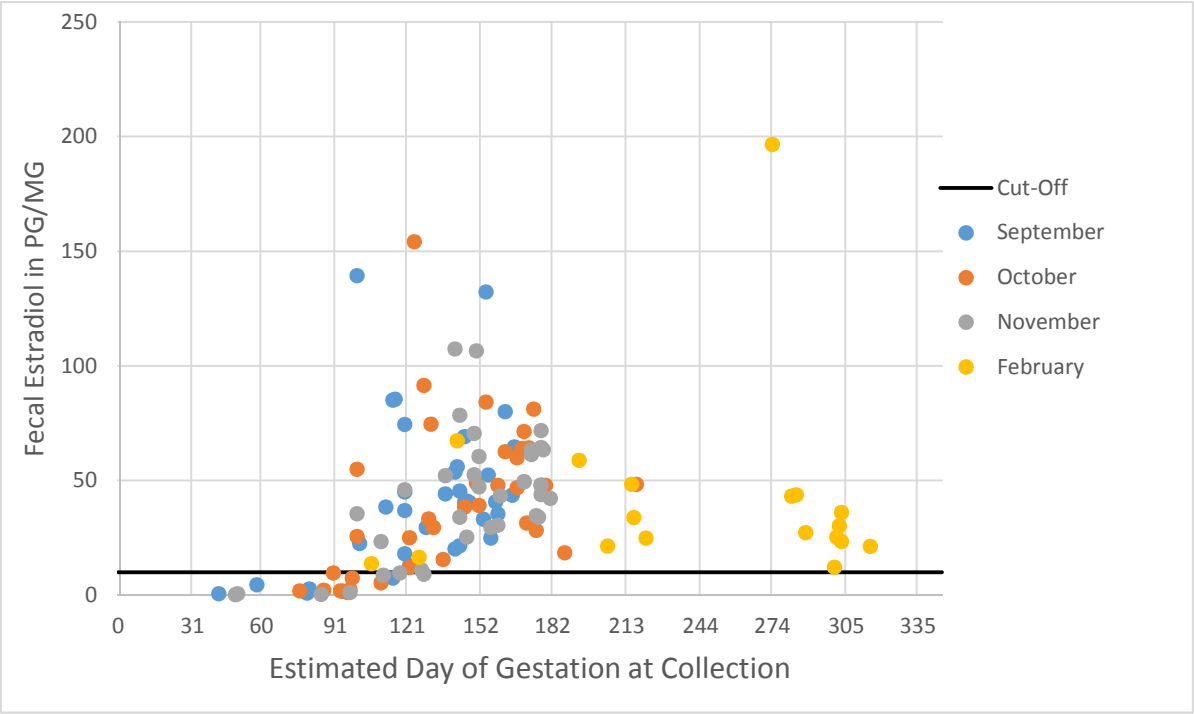


Figure 14. Fecal estradiol concentrations from samples collected from pregnant feral mares in pg/mg. The horizontal line indicates the COC of 10 pg/mg feces.

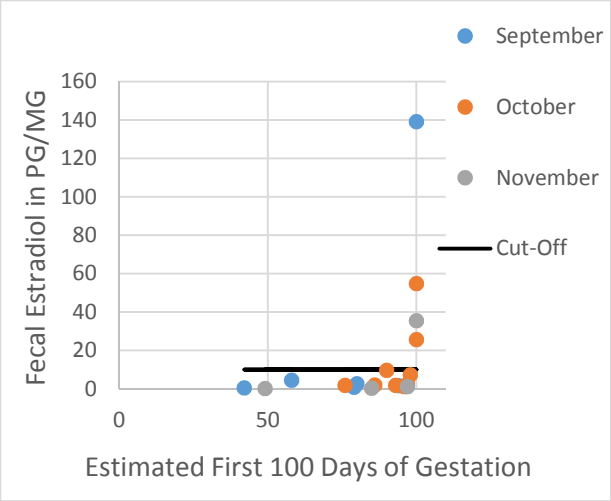


Figure 15A

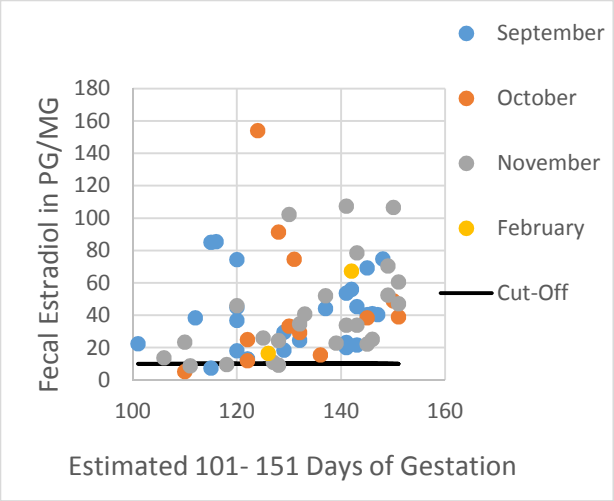


Figure 15B

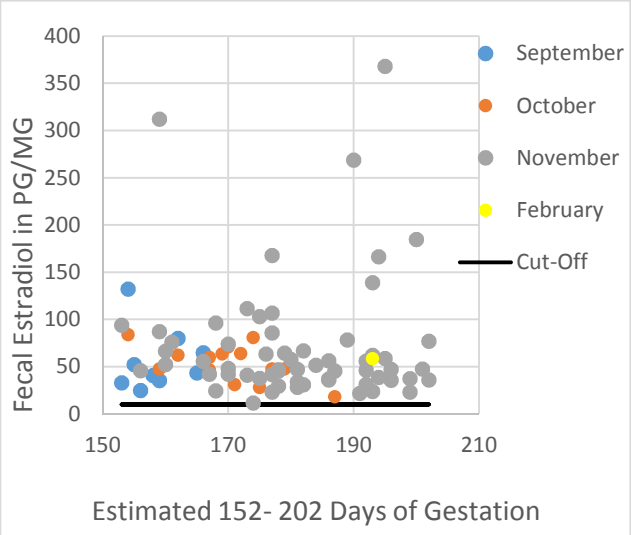


Figure 15C

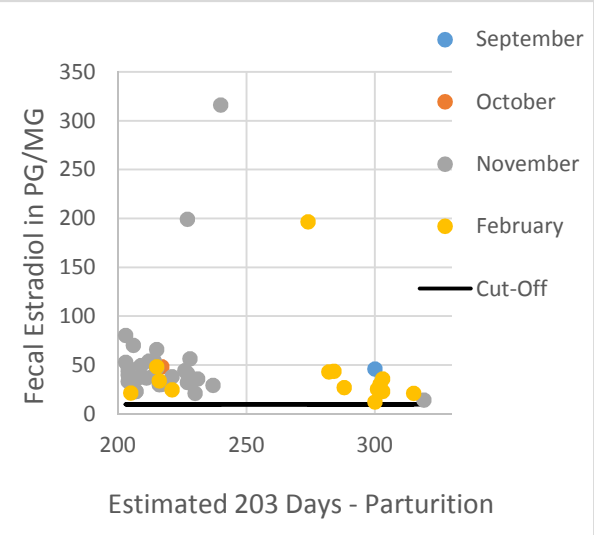


Figure 15D

Figure 15A-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 7. 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

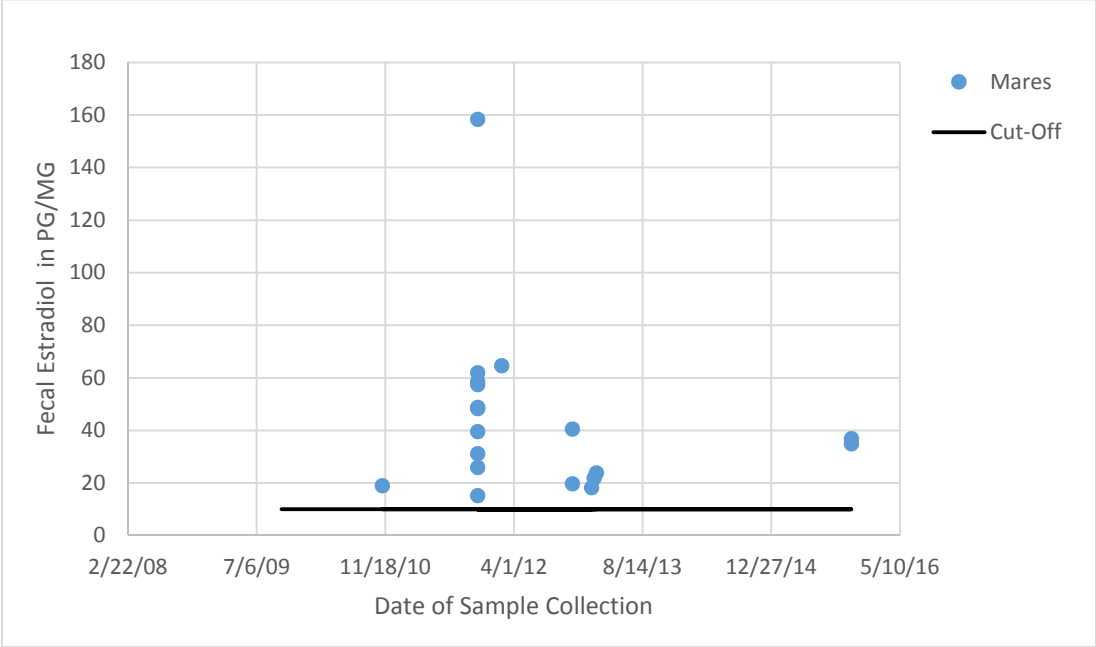


Figure 16. Fecal estradiol concentrations from 22 feral mares who were pregnant at time of collection, but whose foals were not found the following year. The horizontal line indicates the concentration cut-off of 10 pg/mg feces.

Discussion

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. Of the fecal samples taken from pregnant mares during the round-up past 105 days of gestation, 60/62 samples were above the cut-off concentration of 10 pg/mg feces, as noted in Table 4. Conversely, of the 49 serum samples taken post 128, 15 of them were below the designated serum cut-off of 46 pg/mL (Table 4). Additionally, all 19 fecal and serum samples taken from non-pregnant mares at the round-up were below their respective concentration cut-offs (Table 6). 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 9A). 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 10A.

As all sample gestation date estimates were based on a pre-determined estimate of 345 days of gestation length in conjunction with foaling data, 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 5. 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).

As shown in Figure 13B, the treatment effect of GonaCon-Equine can be seen relative to sampling timepoint. After the booster in September 2013, the overall number of samples from non-pregnant GonaCon-Equine treated mares increased. Of the total 121 samples, there were 11 collected in 2010 from non-pregnant GonaCon-Equine treated mares, while in 2015, there were 25 samples

collected. Although these results indicate a possible positive efficacy effect with repeated GonaCon-Equine injections at a four-year frequency, additional research would need to be completed.

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 Figure15A-D shows the overall breakdown by sampling month relative to gestation, it is interesting to note the distribution of samples. As seen in Table 8, 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 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 7, 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 16 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. While it is not known what happened to their foals following sample collection, they appeared to be pregnant at the time of collection.

Table 8. Estimated day of gestation in feral mares relative to the number of samples taken during each collection month. The first number indicates how many of the total for each set surpassed the cut-off concentration of 10 pg/mg.

Estimated Day of Gestation Relative to Sampling Month				
	Estimated Day of Gestation			
	0-100 days	*101-151 days*	*152-202 days*	*203-345 days*
Month	Number of Samples			
September	1 of 6	24 of 25	9 of 9	1 of 1
October	2 of 9	11 of 12	14 of 14	1 of 1
November	1 of 4	22 of 24	73 of 73	30 of 30
February	0 of 0	4 of 4	0 of 0	14 of 14

CHAPTER 4

Conclusions

Through completion of the two studies measuring fecal and serum estradiol, it was found that fecal estradiol measurements indicate pregnancy status both at an earlier average day of gestation (105 days), but also at a lower cut-off concentration (10 pg/mg feces), as compared to serum. Serum estradiol resulted in an average cut-off day of 128 days, and cut-off concentration of 46 pg/mL serum.

Additionally, fecal estradiol concentrations were found to fluctuate less than serum, once the initial cut-off day had passed. In pregnant domestic mares, 258/267 (96.6%) of fecal estradiol samples surpassed both the COD of 105 days, as well as COC of 10 pg/mg feces. Of the nine that failed to surpass the COC, all of them were either within a few days of the COD or parturition. In domestic mares, serum estradiol concentrations in domestic mares resulted in 239/252 (94.8%) samples exceeded COD of 128. Of the 13 that failed to surpass COC of 46 pg/mL, many were scattered throughout gestation, instead of early or late. This trend was also seen in the feral mare samples collected at the roundups in Theodore Roosevelt National Park (THRO), in which 60/62 (96.8%) of fecal samples collected at the roundups surpassed both cut-offs, while only 34/49 (69.4%) serum samples did.

Aside from the fluctuations seen in both feces and serum, the resulting timepoint for definitive diagnosis of pregnancy via fecal estradiol is earlier than what has previously been reported in the literature, with the cuf-off day being roughly two weeks – 2.5 months earlier 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). The results from this study therefore provide a useful, earlier alternate option to the current methods utilized for pregnancy diagnosis in the feral horse.

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APPENDICES

Appendix 1
Colorado State University
Animal Reproduction and Biotechnology Laboratory
Endocrine Laboratory

EXTRACTION OF FECAL ESTROGEN (EXT-FECAL-ESTRADIOL)

Components of Estrogen Hormone Extraction for Fecal Samples

A. Sample
   1. Weight of feces to extract: 10 mg
   2. Reconstituted Extract Dilution Factor 1.0

B. Quality Control (QC) Serum
   1. Low QC: **E_2 LO** Middle QC: **E_2 MED** High QC: **E_2 HI** Solvent Control
   2. Volume to Extract: 0.5g
   3. Reconstituted Extract Dilution Factor: 1.0

C. Solvent
   1. Solvent: **ETHYL ETHER**
   2. Volume Per Tube for Extraction **5.0 ml**

D. Buffer
   1. Buffer: **0.1% PBS-GEL PH 7.0**
   2. Volume to Reconstitute Sample: **0.5 ml** (concentration=1ml/ml)

E. First Extraction
F. Second Extraction
G. Third Extraction
H. Reconstitute

PROCEDURE

A. Extraction Preparation
   1. Freeze feces in -20°C (-22 to -18°C) freezer.
   2. Tape a Kim wipe onto the top of each container of feces.
   3. Lyophilize for 36-48 hr.
   4. Hand grind until fine.
   5. Weigh out approximately 10 mg of feces and place into 16x150mm glass tubes. Record weight for each sample.
   6. Rehydrate with 5ml double-deionized water.
   7. Agitate the slurries for 1 hr(1.0 – 1.5hrs)
   8. Collect approximately 3 ml of supernatant.
   9. Freeze supernatant at -20°C (-22 to -18°C) until second extraction.

B. First Extraction
   1. Aliquot 500 µL fecal supernatant into 16x150mm glass tube.
   2. Add 5mL ethyl ether to each tube.
   3. Vortex for 1 min (1-2 min).
   4. Snap-freeze tubes in a methanol-dry ice bath.
   5. Pour organic phases into clean 16x150mm glass tubes containing 500µL double distilled water for washing.
   6. Vortex tubes for 1 min (1-2 min).
   7. Snap-freeze tubes in a methanol-dry ice bath.
   8. Pour washed organic phases into clean 12x75mm glass tubes.
   9. Dry down under nitrogen.

C. Second Extraction
   Repeat First Extraction steps 2-9.

D. Reconstitute
   1. Reconstitute 12x75mm glass tubes to 0.5 ml with PBS gel.
   2. Vortex for 1 min.
   3. Incubate samples for 18 hr (18 – 20 hr) at 22 °C (20-24°C).
   4. Vortex again and assay.

Appendix 2
Colorado State University
Animal Reproduction and Biotechnology Laboratory
Endocrine Laboratory

EXTRACTION OF ESTRADIOL (EXT-E2)

Components of Estradiol Extraction

A. Sample
   1. Volume of Serum to Extract: **up to 1.0 ml**
   2. Reconstituted Extract Dilution Factor: **1.0**

B. Quality Control (QC) Serum
   1. Low QC: **E2 LO** Middle QC: **E2 MED** High QC: **E2 HI** Solvent Control
   2. Volume to Extract: **1.0 ml**
   3. Reconstituted Extract Dilution Factor: **1.0**

C. Solvent
   1. Solvent: **DIETHYL ETHER**
   2. Volume Per Tube for Extraction: **5.0 ml**

D. Buffer
   1. Buffer: **0.1% PBS-GEL PH 7.0**
   2. Volume to Reconstitute Sample: **1.0 ml** (concentration=1ml/ml)
E. Aliquot Sample
F. First Extraction
G. Second Extraction
H. Reconstitute

PROCEDURE

A. Aliquot Serum Sample
   1. Label 16x150mm glass tubes totaling the number of serum samples.
   2. Label 12x75mm glass tubes with the same numbers.
   3. Label 16x150mm glass tubes and 12x75mm tubes for QC and solvent control.
   4. Aliquot 1.0 ml of serum into 16x150mm glass tubes. Includes QCs.

B. First Extraction
   1. In the extraction hood, add 5.0 ml Diethyl Ether to all tubes.
   2. Vortex for 1 min (1-2 min). Let stand for 5 – 10 minutes.
   3. Freeze in dry-ice and methanol bath.
   4. Pour into 12x75mm glass tubes and dry down in the heating block under nitrogen.

C. Second Extraction
   Repeat First Extraction steps 1-4 using same 16x150mm and 12x75mm glass tubes.

D. Reconstitute
   1. Reconstitute 12x75mm glass tubes to 1.0ml (up to 1.0ml) with PBS-Gel.
   2. Vortex for 1 minute.
   3. Incubate at least 2 h at room temperature or preferably overnight at 4°C (2-6°C).
   4. Vortex again and assay at 200 µL.

Appendix 3
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Animal Reproduction and Biotechnology Laboratory
Endocrine Laboratory

RADIOIMMUNOASSAY FOR 17 B ESTRADIOL-6TME (RIA-E2-6)

Components of Assay
A. Antibody
   1. Antibody Batch Identification **A737**
   2. Dilution of Antibody: **1:400,000**
   3. Diluent: **1:400 Normal Rabbit Serum(NRS) in PBS-EDTA**
   4. Volume Per Tube in Assay: **200 µL**

B. Iodinated Preparation
   1. Iodinated Preparation Identification: ^{125}**I-ESTRADIOL-17BETA-6-TME**
   2. Approximate Counts Per Minute/100 µL: **30,000 IN PBS-GEL**
   3. Volume Per Tube in Assay: **100 µL**

C. Standards
   1. Standards Batch Identification: **E2-CSU-S15**
   2. Stock: **USP 1250008 Lot# R025F0**
   3. **0.2** ng/ml
   4. Number of Points in Curve: **6**
   5. Dilution Factor: **0.4**

D. Secondary Antibody
   1. Secondary Antibody Identification: **A1039** Goat Anti-Rabbit (GAR)
   2. Dilution 1:**100** in PBS-EDTA
   3. Volume to Assay: **200 µL**

E. Quality Control (QC) Sera
   1. Low QC: **E2 LO** Middle QC: **E2 MED** High QC: **E2 HI** Solvent Control
   2. Reconstituted Extract Dilution Factor: **1.0**
   3. Volume to Assay: **80 µL**

F. Sample
   1. Reconstituted Extract Dilution Factor: **1.0**
   2. Volume to Assay: Up to 200 µL

G. Buffer
   1. PBS-Gel 0.1% pH 7.0
   2. Volume to Assay: Appropriate amount to bring Sample + Buffer to 500 µL

H. Incubation: **24 – 72 hours minimum at 4°C**

PROCEDURE

A. Assay Procedure
   1. To glass 12x75mm tubes add up to 200 µL of extracted sample/QC or standard and appropriate amount of buffer to bring column of sample/QC or standard + buffer to 500 µL. Add 500 µL buffer to Non-Specific Binding (NSB) (tubes 4 – 6) and maximum binding tubes (tubes 13 – 15).
   2. Add 100 µL ^{125}I-E2-6TME to all tubes.
   3. To the NSB tubes add 200 µL NRS at 1:400 dilution.
   4. Add 200 µL E2-6TME antisera to all tubes except total count (TC) tubes (tubes 1-3) and NSB tubes.
   5. Vortex.
   6. Incubate in 4°C for 24 hours.

B. Addition of Secondary Antibody
   1. At least 24 hours after adding the radioactive tracer to the assay, add 200µL ARGG to all tubes except the TC tubes.
   2. Vortex.
   3. Return to the 4°C incubator for 72 hours.

C. Pouring off Assay
   1. At 72 (± 8) hours after adding GAR, load all of the tubes, except TC tubes, into the centrifuge carriers.
   2. Add 3 mL cold phosphate buffered saline to each tube.
   3. Balance carriers.
   4. Centrifuge at 2500 rpm for 30 minutes.
   5. Pour off the supernatant into liquid waste containers.
   6. Blot the tube rims gently.

D. Counting
   1. Determine the efficiency/background of gamma spectrometer using calibrated ^{125}I sources.
   2. Count the radioactivity associated with the pellet in a gamma spectrometer.
   3. Reduce raw counts onto 3 ½" floppy disk.

E. Process Data
   1. Using 3 ½ "floppy disk, transfer raw counts from gamma spectrometer to computer containing the program RIANAL.
   2. Save as counts file (.ct) which corresponds to protocol file (.pt).

F. Analyze Data
   1. Generate results of assay using RIANAL program.
   2. Save Analysis onto private Drive (U:)

G. Quality Control
   1. Record quality control information in the QC book.
   2. Compare results of QCs from assay with values from previous assays. If results of QCs from current assay differ more than 2 standard deviations from the mean of previous assays, the assay is to be rerun.

H. Assay Review
   1. Non-Detectable level must be determined for all assays that contain non-detectable values in the results.
   2. All completed assays must be reviewed by laboratory supervisor before sending out results.