INDUCTION OF EARLY CYCLICITY USING ESTRADIOL CYPIONATE AND SULPIRIDE ON SEASONALLY ANESTROUS MARES

by

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ABSTRACT

This study was designed to evaluate strategies to induce early ovulation in anestrous mares. Thirty-two seasonally anovulatory mares were randomly placed into 4 treatment groups based on their reproductive status. Anestrous (n = 11), transitional (n = 8), and progesterone pre-treated (n = 9) groups were treated in January with 50 mg of estradiol cypionate (ECP) followed 24 h later with 3 g of sulpiride. A control group (C, n = 4) was administered the treatment carrier. Ovaries were scanned every 2 to 3 d via transrectal ultrasound for 60 d following treatment. Day of first and second ovulation was recorded. Embryos were transferred to 10 mares after at least 2 estrous cycles. Blood samples were analyzed for luteinizing hormone (LH) and prolactin (PRL). There was an effect of treatment ($P < 0.001$) on mean number of days to first ovulation. Mean number of days to first ovulation was less ($P < 0.001$) in all treated groups than C. There was an effect of treatment on the number of mares that achieved first ovulation ($P < 0.001$) and second ovulation ($P = 0.018$). Of 28 treated mares, 23 (82.1%) ovulated within 22 d of treatment. Of 23 mares that had a first ovulation, 15 (65.2%) had a second ovulation within 43 d. There was an effect of the treatment-by-day interaction on plasma mean PRL ($P < 0.001$) and LH ($P = 0.021$) concentrations. Ten embryos were transferred across the 3 ECP-sulpiride treated groups with 70% of mares having confirmed pregnancy at 60 d.

Key Words: anestrus, anovulatory, cyclicity, estradiol cypionate, mares, sulpiride.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Anestrous mares pose a unique challenge for the equine breeding industry due to their natural cycling relative to the January 1 birthdate that is standard across many breeds. Many people who breed horses strive to produce foals as close to, but not before January 1, since foals born earlier in the year have a physical maturity advantage over foals born later, yet are considered the same age classification. Hence, breeding mares earlier in the year can be advantageous for offspring to help maximize performance and related prices obtained in the sale ring (Squires, 2008). Most mares are in anestrus early in the year as horses are a seasonally polyestrous cycling species. Consequently, people scheduling the breeding of horses often work to circumvent the mare's natural seasonal cycling. In the northern hemisphere, mares are long-day breeders that naturally cycle from April to October. Since the mare's normal gestation period is 11 mo, in order to have a birthdate in January, a mare must conceive as early as February of the previous year. While various strategies for inducing early ovulation have been developed, most options for managing anestrous mares are far from optimal. For example, there are limited strategies that are economical and labor efficient for mares housed in large herds such as recipient mares used for embryo transfer. Embryo transfer offers potential benefits for breeders, though effective management of recipient mares is crucial for

maximum success. Thus, the equine breeding industry would benefit from a simple and economical approach that can be applied to herds. The objective of this study was to stimulate ovarian activity early in the calendar year, and decrease the erratic estrous cycling that would be typical of the transitioning mare from anestrus to normal cycling.

There are a variety of hormonal regimens and treatments that have been attempted to hasten ovulation to the beginning of the year. One of the most promising approaches has been the use of a dopamine antagonist to stimulate an increase in prolactin (PRL). Prolactin was once thought to only be involved in lactation, but research has discovered that PRL has other reproductive roles in the body (Thompson and Oberhaus, 2015). Prolactin's role in the reproductive system has generated research interest on how to potentially utilize the hormone. Horses have higher PRL concentrations during the breeding season in comparison to the mare's anestrous period (Thompson et al., 1986a and b). Prolactin appears to exert action directly on the ovarian follicle as PRL receptors have been reported to be located on equine follicular cells (Oberhaus et al., 2015). Although no PRL releasing factor has been identified in horses, PRL homeostasis has been observed to be primarily regulated by dopamine. Thus, the use of dopamine antagonists have offered an approach to potentially manipulate the estrous cycle of mares.

Sulpiride is a dopamine antagonist that has been reported to increase PRL concentrations and potentially hasten ovulation (Besognet et al., 1997; Mari et al., 2009; Panzani et al., 2011). Kelley et al. (2006) and Mitcham et al. (2010) reported that the addition of estradiol greatly enhanced the effect of sulpiride alone, and researched the most effective combination and dosage of estradiol and sulpiride. Oberhaus et al. (2017,

2018) investigated the differences between individual mare responses to a combined estradiol-sulpiride treatment and the mechanisms of how the hormone combination changed the mare's cycle. Further research of the combined estradiol-sulpiride treatment would be beneficial in further determining the effectiveness and application of the treatment in a commercial setting such as an embryo-transfer program. Previous research has indicated that a combined estradiol and sulpiride combination will shorten the interval from anestrus to normal cyclicity (Kelley et al., 2006; Oberhaus et al., 2017) Thus, the objective of this study was to induce early cyclicity in anestrous mares with administration of estradiol cypionate (ECP) and sulpiride utilizing a unique dosage and timing protocol.

CHAPTER II

REVIEW OF LITERATURE

Natural Cycling

The horse is a seasonally polyestrous species. Normal cycling in mares starts in the spring as a result of an increase in the length of daylight, temperature, and availability of food. The natural breeding season in mares in the northern hemisphere is from April through October, and increased photoperiod (or day length) is one of the primary factors of transitioning from winter anestrus to the breeding season (Ginther, 1979). The retina in the eye detects changes in photoperiod length and those changes are then signaled to and processed by the pineal gland. Melatonin is released by the pineal gland during periods of darkness (Ginther, 1979). Melatonin appears to have a role in regulating reproductive seasonality in the mare, but the exact mechanism by which melatonin exerts action in the mare is unclear. In the spring, as the hours of light increase, melatonin secretion decreases and GnRH secretion increases causing an increase in luteinizing hormone (LH) secretion, which initiates the onset of cyclicity (Oberhaus and Paccamonti, 2013).

Natural cycling varies among individual mares with many factors affecting individual cycles. Age, nutrition, climate, and management may all factor into when and how a mare cycles. The numerous factors that regulate cyclicity in mares can be challenging to people breeding horses when attempting to produce a foal early in the

year. Mares have, on average, an 11-mo gestational length. Thus, in order for a foal to have a birthdate early in the calendar year, there must be a corresponding date of conception. For example, in order to have a foal born in January it must be conceived in February of the previous year.

Estrous Cycle in the Mare

The estrous cycle consists of two phases: estrus and diestrus. The length of the estrous cycle can be defined as the length of time from one ovulation to the subsequent ovulation (Brinsko et al., 2011). The estrous period is also known as the interovulatory interval. The average length of estrus, diestrus, and the estrous cycle in horses are 7, 15, and 22 d, respectively (Ginther, 1979). The length of estrus is more variable and dependent on environmental factors while diestrus is consistent in length (Ginther, 1979, Brinsko et al., 2011).

The regular pattern of the estrous cycle relies on a delicate balance between hormones. There is a series of signals between the retina and the ovary that regulates the mare's cycle. The retina receives stimuli from light that is relayed by nerves to the pineal gland, which regulates the secretion of melatonin. Melatonin influences the neurosecretory cells in the hypothalamus that produce gonadotropin-releasing hormone (GnRH). As melatonin decreases, GnRH increases. Gonadotropin-releasing hormone is transported to the anterior pituitary (AP), and the two gonadotropins (FSH and LH) are stimulated and released into systemic circulation, which exert their effect on the ovaries through receptors. Receptors for FSH and LH are located on granulosa and thecal cells, respectively (Irvine and Alexander, 1993). Once at the ovary, FSH is responsible for follicular recruitment, and LH is responsible for follicular maturation, production of

estrogen, ovulation, and luteinization (Brinsko et al., 2011). Estrogen production during the follicular phase results in estrus. Estrogen produced by the mature follicle in the presence of low circulating progesterone concentration has a positive feedback on LH release at the AP. Immediately before ovulation, LH surges to stimulate ovulation of the mature follicle. Once ovulation occurs, progesterone produced by the corpus luteum suppresses LH secretion from the AP. Maximum circulating progesterone from the corpus luteum is reached 6 d after ovulation (Brinsko et al., 2011). If the mare is not pregnant, the corpus luteum is terminated between 13 to 16 d after ovulation. Luteolysis results in a decline of circulating progesterone and ends the suppression on LH and FSH secretion which restarts the follicular phase.

Hypothalamic-Pituitary Axis

The hypothalamus and AP together are responsible for coordinating a magnitude of reproductive organ functions and plays a substantial role in the chain of commands that regulate seasonality and normal cycling. Reproductive rhythmicity is coordinated through the hormones from the hypothalamus, pituitary, and reproductive organs (Ginther, 1979). The hypothalamic-pituitary axis not only coordinates the reproductive tract with other systems of the body, but also the external cues from the environment that influence the reproductive system as well. Internal and external stimuli are received by the pineal gland and the hypothalamus. GnRH secretion from the hypothalamus stimulates production of GnRH receptors on gonadotrope cells in the AP (Clayton, 1989). Irvine and Alexander (1993) reported that estradiol can induce the production of more GnRH receptors. The pulsatile secretion patterns of GnRH changes throughout the cycle. The change in GnRH pulses control the differential release of the

gonadotropins (Ginther, 1979). Production and secretion of LH correspond with rapid GnRH secretion pulses. On the other hand, FSH secretion responds to slow pulses every 6 h (Irvine and Alexander, 1993). During estrus, GnRH pulses are increased to 2/h and LH secretion is high while FSH secretion is low. In contrast, during diestrus, GnRH pulses occur an average of 2/d, causing FSH secretion to increase and LH secretion to decrease (Irvine and Alexander, 1993). Aside from GnRH, other regulatory factors come from the hypothalamus to control production and secretion of pituitary hormones that affect reproductive function such as FSH, LH, PRL, and adrenal corticotropin hormone.

Follicular Growth and Ovulation

The physical development aspect of follicles from formation to ovulation is referred to as folliculogenesis. The mare, like other mammalian females, is born with a fixed number of oocytes that have the potential to mature (Ginther, 1979). Follicles are an oocyte surrounded by layers of cells. Each estrus typically has one or two follicles that progress into mature follicles ready for ovulation. A group of follicles grow during the luteal phase, and concentration of FSH increases resulting in an increase in small follicles (Ginther, 1979). The follicles greater than 30 mm diameter grow until they either ovulate or regress. Some mares can have 2 follicular waves within one cycle, the second follicular wave sometimes results in a diestrus ovulation.

In light breeds of horses, follicle diameter at ovulation typically ranges from 30 to 50 mm (Brinsko et al., 2011). Most mares ovulate within 48 h before the end of estrus. Time of ovulation can be predicted via rectal ultrasound examination. Degree of uterine edema, cervical relaxation, size and softness of the dominant follicle, characteristics of the follicular wall, and fluid in the uterus can all help predict timing of ovulation. The

mare's ovulatory LH surge is prolonged as compared to other species, with concentrations rising gradually throughout estrus to peak the day after ovulation (Ginther, 1979).

Anovulatory Season

In the winter months as day lengths shorten, a mare will stop cycling normally and go into an anestrous period. The management of mares can be challenging in the anestrous period because of the unpredictable and inconsistent nature with transitioning in and out of cyclicity. Every mare's anestrous period is somewhat unique and a single mare's anestrous period may vary annually (Brinsko et al., 2011). As an example, a mare could stop cycling in November of one year, December the next year, or cycle through an entire year without going into anestrus. About 30% of mares show ovulatory cycles throughout the winter season (Aurich, 2011).

The anovulatory season in mares is characterized by 3 phases (Ginther, 1979). The first phase refers to the receding phase that occurs after the last ovulation of the year and is sometimes referred to as "fall transition". During fall transition, changes in pituitary and ovarian hormones subsequently alter normal follicular growth. Irvine et al. (2000) reported that failure to ovulate in the autumn may be associated with decreased LH concentrations. They reported that a LH surge terminated sooner in the last cycle rather than in the second to last cycles, and failed to occur when mares were in anestrus. The gradual reduction of LH leads to an inactive state, which is the second phase. The inactive phase can also be called deep anestrus in which mares have no follicular growth. Lastly, is the resurging phase or "spring transition". Management of the spring transition period from anestrus to normal cyclicity can require more effort because the first ovulation of the year can be difficult to predict and varies among mares. Some mares

have longer transition periods than others, but length of transition usually ranges from 30 to 90 d (Aurich, 2011).

Supplemental Light

Supplemental light treatment to mares is one of the most effective methods for hastening cyclicity in anestrous mares in the spring, however, there are some challenges associated with managing the application of supplemental lighting. The use of supplemental light was first reported in the 1940's, but extensive research was not performed on how light affects equine reproduction until the 1970's (Ginther, 1979). Research has indicated that for light therapy to be effective in hastening cyclicity in the northern hemisphere, mares must start light exposure in December at the latest (Brinsko et al., 2011). Photoperiod stimulation must start 60 to 70 d before the desired ovulation time for a total of 14 to 16 h/d including natural and artificial light (Ginther, 1979; Brinsko et al., 2011). Management of supplemental light therapy has challenges when applied to mares housed in herds such as recipient mares for embryo transfer. A 200-watt incandescent light bulb was reported to produce enough light exposure if the mare stands within 2 to 3 m of the bulb (Brinsko et al., 2011). During the day, stalls should have windows allowing the same amount of exposure of natural light. Shadows or unlighted areas can preclude adequate light from the intended goal. Overall, the process of providing supplemental light can lead to challenges resulting in a timely process that can be costly and labor intensive.

Other Hormonal Regimens to Hasten Ovulation

There are a variety of hormonal approaches to induce early cyclicity in anestrous mares. One example is treatment with GnRH which has been shown effective in

stimulating some seasonally acyclic mares to ovulate (Oberhaus and Paccamonti, 2013). Alexander and Irvine (1996) replicated the cyclic changes in GnRH pulse frequency to induce ovulation. Injections of GnRH were given every 8 h for 21 d to stimulate folliculogenesis. After 21 d, the GnRH injections were administered hourly. All mares ovulated and 75% established a corpus luteum. Even so, administering multiple GnRH injections to a large group of mares is not convenient and can be costly. In many species, exposure to continuous GnRH concentrations reduces gonadotrope responsiveness to further stimulation. However, in the mare, continuous stimulation will still lead to ovulation (Alexander and Irving, 1996). Many GnRH analogs are available in long-acting, continuous formulations and packaging that can be implanted subcutaneously. Implants of GnRH, however, can be expensive and overall, the success rate of GnRH and GnRH analogues are dependent on the depth of the mare's anestrous period. Oberhaus and Paccamonti (2013) reported that mares that received continuous infusion of GnRH beginning in February returned to acyclicity after GnRH treatment stopped. Overall, GnRH can require multiple injections and be dependent upon injection timing. The method has yet to be proven practical and cost effective for horse breeders.

Another hormonal strategy to induce cyclicity has involved the use of progestins. Nevertheless, progesterone treatments have been reported only effective in late-spring transitional mares (Oberhaus and Paccamonti, 2013). Van Niekerk et al. (1973) first reported the use of progestins in transitional mares. Injections of 100 mg of progesterone in oil administered daily for 7 d caused mares in late transition to cease signs of estrus within 2 d and ovulate 6 to 7 d after administration of progesterone stopped (Van Niekerk et al., 1973). Later studies were conducted using altrenogest, a synthetic progestin. The

altrenogest resulted in similar effects on late transitional mares (Webel and Squires, 1982). However, both methods of progesterone treatment have proved to be ineffective on mares deep in anestrus or early transition (Squires, 2008; Oberhaus and Paccamonti, 2013). Since daily progesterone injections are time consuming and inconvenient, administration of a single injection of a controlled release preparation of progesterone and estradiol that released the hormones over 12 to 14 d was explored. The controlled release method resulted in successful stimulation of ovulation only in late transitional mares or mares with follicles greater than 30 mm (Burns et al., 2006).

Lastly, pituitary extracts such as equine FSH or LH have been used successfully to induce normal cyclicity in transitional mares (Squires, 2008). In 2004, Niswender et al. reported that transitional mares given commercial equine FSH twice daily resulted in 8 of 10 mares ovulating after 5 d of administration. Schauer et al. (2011) administered equine LH to mares in early transition. Once a follicle reached 32 mm, human chorionic gonadotropin (hCG) was administered to induce ovulation. Although follicular growth resulted, the follicles produced were unresponsive to hCG. Furthermore, once treated mares ovulated, they returned to an anovulatory transition state (Schauer et al., 2011). Most protocols also require at least 2 injections per day for multiple days for the treatment to be effective (Squires, 2008).

Prolactin

Prolactin is a protein hormone produced and secreted by the AP that has been traditionally considered to have a predominant role in lactation, though appears to have a role in the mechanism of transitioning in and out of cyclicity as a gonadotropin. Stricker and Grueter first described PRL in 1928 when they observed a stimulatory activity of

extracts of bovine adenohypophysis on rabbit mammary glands (Hadley, 2000). The term "prolactin" was then used in 1993 by Riddle who reported the stimulation of pigeon crop sacs by a similar extract (Thompson, 2015). Prolactin became the term widely used due to the hormone's milk-inducing effects. Equine PRL was first isolated and partially characterized in 1979 (Thompson and Oberhaus, 2015). Prolactin in the horse is similar to PRL in other species, being a single chain protein consisting of a 199 amino acid unit. However, equine PRL is unique in that 2 disulfide bridges are present in contrast to 3 bridges in other species (Ginther, 1979). Although equine PRL is 93 to 95% homologous to porcine PRL, the minor differences appear to be enough to elicit antibody production in the mare (Thompson et al., 1997). Equine PRL is 94, 78, 76, and 75% homologous to canine, human, bovine, and ovine PRL, respectively (Thompson and Oberhaus, 2015); suggesting that the structural similarity could potentially result in similar functionality across species.

Prolactin is produced by lactotroph cells of the AP and is released in pulses similar to LH and FSH (Ginther, 1979). Lactotropes make up 6.5 to 16.5% of cells on the equine AP (Rahmanian et al., 1997). The lactotroph cells can be divided into two specific types. Type-I lactotroph cells are larger with a denser secretory granule while type-II lactotroph cells are small with dense polymorphic granules that are located mostly in peripheral areas of the cytoplasm. Rahmanian et al. (1997) reported a subset of cells, called mammosomatotropes, in the equine AP that contained growth hormone and PRL in the same secretory granules.

Prolactin has a wide range of biological actions in the bodies of many species. As one of the most pleiotropic hormones known, PRL's function in one species is not the

same across all species (Langan et al., 2018). Research data in rats, suggest that PRL could stimulate the corpus luteum because of PRL's importance in progesterone production of the corpus luteum (Ginther, 1979). The evidence of this luteotropic role is not supported across all species, despite the presence of PRL receptors on the luteal cell's membranes in most species.

Research on PRL in horses has been performed on mares, geldings, and stallions. In the horse, season, exercise, sexual excitement, stress, and feeding can affect PRL secretion (Thompson et al., 1997). Prolactin has been observed to be associated with the rise in seminal volume after sexual stimulation of stallions (Thomson et al., 1996). Colborn et al. (1991b) reported that PRL and cortisol concentration increases after sexual excitement and acute exercise in the summer when PRL is already high, as well as in the winter (Colborn et al., 1991a).

Prolactin increases during the ovulatory season in relation to increasing day length (Ginther, 1979; Brinsko et al., 2011). Fitzgerald and McManus (2000) reported that increased melatonin during short photoperiod decreased PRL concentration in mares. Since PRL appears to have a role in seasonality, research has been performed to determine PRL's function with regard to the reproductive system, hair shedding, metabolism, and various endocrine regulation. Nequin et al. (1993) used ovine PRL to stimulate follicular growth in seasonally anestrous mares. Thompson et al. (1997) performed various experiments on anestrous mares treated daily with recombinant porcine PRL, one of which monitored hair shedding. Treatment with recombinant porcine PRL increased hair shedding within 14 d, peaking at d 28, before decreasing (Thompson et al., 1997). The presence of antibodies was confirmed by d 28 and thus explained the

reversal of the induced hair shedding. Furthermore, the treated mares had patches of winter hair coat 3 mo later indicating that the antibodies persisted long after the last treatment of recombinant porcine PRL. The antibodies evidently neutralized the normally increasing amounts of endogenous PRL normally secreted at that time of year (Thompson et al., 1997). Thompson et al. (1997) reported that LH concentrations by mares treated with PRL was minimal compared to LH concentrations attained in estrous mares during the breeding season. Therefore, although time of ovulation was hastened in the treated mares of this experiment, PRL was speculated to affect ovulation through direct follicle maturation or the ovulation process rather than stimulation of LH production (Thompson et al., 1997).

Receptors for PRL have been localized on equine ovarian follicular cells (Oberhaus et al., 2015), which is an indication that PRL can exert action directly on the ovarian follicle. These authors determined that PRL receptors were located on equine ovarian follicle cells, and the number of receptors varied through different stages of follicular growth and the estrous cycle.

A primary PRL releasing factor has not been identified, rather PRL homeostasis appears to be regulated through many hypothalamic, systematic, and local factors acting as stimulators. Dopamine reportedly inhibits PRL secretion (Ben-Jonathan and Hnasko, 2001). Various stimuli can have an effect on PRL secretion including season, exercise, stress, thyrotropin-releasing hormone, meal feeding, estrogen treatment, dopaminergic and antidopaminergic agents, as well as antiopiodergic agents (Alexander et al., 2004). Steroids have also been reported to affect PRL concentrations. Thompson et al. (1991) reported a 5-fold stimulation of PRL storage in the pituitary by estradiol treatment in

ovariectomized mares, whereas the effect on PRL plasma concentrations was much less. Aurich et al. (1995) reported an increase in PRL concentration in ovariectomized mares after administration of estrogen plus naloxan, an opioid antagonist. In addition, paracrine and autocrine interactions within the AP alter PRL regulation (Freeman et al., 2000). In sheep, a disconnection of the hypothalamus and AP resulted in unaltered PRL pulse frequency suggesting that PRL fluctuations are generated within the AP of this species (Thomas et al., 1986).

Dopamine and Dopamine Antagonists

While the complete control of PRL secretion is not fully understood, dopamine appears to have an important role in regulating PRL. Dopamine belongs to a class of neurotransmitters known as catecholamines. Derived from tyrosine, dopamine is primarily synthesized in the central nervous system, but there is also evidence of production in the adrenal medulla as well as non-neuronal tissue such as the pancreas and AP (Ben-Jonathan and Hnasko, 2001). Dopamine can act either as a neurohormone or a neurotransmitter (Hadley, 2000). Catecholamines act as classical neurotransmitters while in the brain. Dopamine, however, is not limited to communication between neurons within the synapse. As a neurohormone, dopamine is a chemical messenger released into the bloodstream by nerve cells (Hadley, 2000). The presence of dopamine in circulation enables communication with distant target organs. Dopamine has many effects in the body, but has foremost influence on the reproductive system through regulation of PRL. Dopamine was established as the primary inhibitor of the AP lactotrophs in the 1970s and has been the regulator of PRL gene expression and release (Ben-Jonathan and Hnasko, 2001). Lactotropes removed from hypothalamic input secrete large amounts of PRL

(Hadley, 2000). Therefore, when dopamine from the hypothalamus is inhibited, PRL from the AP is usually increased

Dopamine also appears to play a role in seasonality. Melrose et al. (1990) conducted a study on dopamine in the cerebrospinal fluid (CSF) of horses. These research authors concluded that CSF dopamine concentrations in horses are dependent upon the presence of gonads. Only gonadally intact mares had a decline in CSF dopamine during the ovulatory season, whereas, ovariectomized mares had no variation in concentrations between seasons (Melrose et al., 1990). There was also an inverse relationship between CSF dopamine concentrations and LH. High concentrations of LH were observed during cycling periods and low concentrations were observed during the anestrous season. In contrast, CSF dopamine was increased during anestrus and decreased once the ovulatory season began (Melrose et al., 1990).

One common dopamine antagonist is sulpiride. Sulpiride, N-[1-ethyl-2-pyrrolidylmethyl]-2meth-oxy-5-sulphamoyl benzamide, is a benzamide substitute. Sulpiride is unrelated chemically to other dopamine receptor antagonist neuroleptics of the phenothiazine, butyrophenone, and thioxanthene groups (O'Connor and Brown, 1982). The difference between sulpiride and the classical neuroleptics has been useful in developing the difference between dopamine receptors. Kebabian and Calne (1979) reported that dopamine has two receptor subtypes, D-1 and D-2. The subtype involved with PRL secretion from lactotropes is D-2 (Munemura et al., 1980; Creese et al., 1983). The D-1 and D-2 pathways take different routes through the basal ganglia, and the dopamine receptors also have different properties (Keeler et al., 2014). The dopamine receptors are a family of G protein-coupled receptors to which extracellular dopamine

binds leading to a response. Generally, when dopamine binds to a D-1 receptor, dopamine functions in an excitatory fashion and in contrast the D-2 receptor response behaves in an inhibitory fashion. Dopamine release can be both tonic, continuous output in the system; and phasic short term burst activity. The D-2 receptors have a high affinity for dopamine and are more sensitive to changes in tonic dopamine concentrations. In comparison, D-1 receptors have relatively low affinity for dopamine and are preferentially sensitive to phasic changes in dopamine activity (Keeler et al., 2014). The D-1 receptors also increase intracellular cAMP activity, while D-2 receptors inhibit cAMP accumulation (Ben-Jonathan and Hnasko, 2001). Sulpiride is selectively an antagonist of D-2 receptors. Sulpiride, in comparison to other classical neuroleptics, has a very low lipid solubility and penetrates the brain poorly after peripheral administration (O'Connor and Brown, 1982). Sulpiride preferentially accumulates in cerebral areas where the blood brain barrier is ineffective such as the AP. This explains sulpiride's potent PRL stimulatory property and sulpiride's relatively weak effects in behavioral models (O'Connor and Brown, 1982). Sulpiride and domperidone are more commonly used in research as they have a low prevalence of side effects. In contrast, metoclopramide and perphenazine can have antidopaminergic actions (Besognet et al., 1996).

The majority of evidence shows that when dopamine is inhibited during late anestrus, the first ovulation of the year is hastened (Brinsko et al., 2011). Johnson and Becker (1987) were the first to report that sulpiride increased PRL concentrations in the mare in July. Besognet et al. (1997) reported that daily administration of sulpiride to anestrous mares significantly advanced the day of first ovulation by 33 d and increased

PRL concentrations when compared to control. Besognet et al. (1997) evaluated bred mares following their first ovulation of the year, and fertility rates for treated and control mares were similar. The establishment of pregnancy, however, was hastened for the treated mares. Similarly, Brendemuehl and Cross (2000) administered domperidone orally and achieved a mean date of ovulation 78 d earlier than the control. Duchamp and Daels (2002) observed that environmental factors such as photoperiod, temperature, nutrition, and body condition could influence the impact of sulpiride treatment. Sulpiride and light treatment were combined in a study with 44 anestrous mares placed in pairs of similar reproductive history, age, and body condition (Duchamp and Daels, 2002). The mares were housed in stalls and were subjected to 14.5 h of light daily beginning on January 10. In addition, 22 mares received daily sulpiride injections beginning in January until the day of ovulation or for a maximum of 21 d. Of the mares treated with sulpirde, 86% ovulated before their untreated pair and the interval from treatment to first ovulation was shortened by 16.7 d (Duchamp and Daels, 2002). Mari et al. (2009) compared the effectiveness between 2 dopamine D-2 receptor antagonists: sulpiride and domperidone. The first ovulation was reported to be hastened in the sulpiride group, but not in the domperidone treated group. Therefore, time of pregnancy was established with the group receiving sulpiride treatment before domperidone and control groups (Mari et al., 2009). Pansani et al. (2011) treated transitional mares in the spring (after detection of 25 mm follicles) with 1 mg/kg BW/d sulpiride, and reported a significant hastening of ovulation. On the other hand, Dondadeu and Thompson (2002) observed no early ovulation after daily sulpiride treatment for 31 d. Although there was an increase in PRL concentration

in treated mares, sulpiride treatment did not stimulate LH or FSH secretion, or ovarian activity under the conditions of the experiment (Dondadeu and Thompson, 2002).

Use of Estradiol and Anti-dopaminergic Compounds

To further increase efficacy of the approach of using a dopamine antagonist to hasten the first ovulation, other factors involved with mare seasonality was added to the treatment. Kelley et al. (2006) conducted research using estradiol benzoate to observe the interaction with sulpiride and domperidone. Inadequate estrogen in seasonally anestrous mares was hypothesized to be the reason for less than ideal PRL and ovarian responses to sulpiride in previous experiments. Estrogen is reported to be a potent regulator of PRL in rodents; however, estrogen has little acute effects on PRL in humans (Ben-Jonathan and Hnasko, 2001). Thompson et al. (1991) reported an increase in PRL concentrations after administration of estradiol in ovariectomized pony mares in the summer. In the research by Kelley at. al. (2006), treated mares received 10 injections of estradiol benzoate every other d before sulpiride administration on d 11. Mares that were pretreated with 10 injections of estradiol ovulated 45 d earlier than mares receiving sulpiride alone. Pretreated mares also had increased PRL and LH concentrations. Frequent blood sampling around the time of sulpiride injections indicated that mares treated with sulpiride alone had a decreased PRL response after subsequent injections. Thompson et al. (1997) reported similar decline in PRL responses after multiple injections of sulpiride. Sulpiride alone was concluded to stimulate secretion of PRL from the pituitary gland, but did not stimulate production in the long-term, and adding estradiol to the treatment increased PRL.

Thompson et al. (2008) determined that a single injection of 100 mg ECP yielded more consistent PRL responses. Mitcham et al. (2010) conducted a few experiments to determine the effectiveness of several protocols for a single injection of ECP combined with a single injection of domperidone. Various combinations and dosage of ECP, domperidone, and progesterone were evaluated. The results indicated that ECP combined with domperidone resulted in more ovulations than domperidone alone, but the addition of progesterone to the combination did not change the response. There also appeared to be no difference between doses of 75, 100, or 150 mg ECP and no difference between doses of 1.5 or 3 g domperidone (Mitcham et al., 2010). Mitcham et al. (2013) also compared domperidone and sulpiride, and observed that 1.5 g sulpiride in a slow-release oily carrier had a greater PRL response than 1.5 g domperidone in biodegradable microparticles.

Oberhaus et al. (2017) treated 23 anestrous mares in January with 50 mg ECP on d 0. Ten of the mares received 3 g sulpiride on d 1 and 13 mares on d 6. The study was intended to evaluate factors that may determine an individual mare's response to the treatment. Mares that responded to the treatment had higher body condition scores, body weight, plasma concentration of insulin and leptin, and higher LH concentrations after a GnRH challenge when compared to nonresponding mares (Oberhaus et al., 2017). Sulpiride given on d 1 or 6 did not affect the degree to which PRL concentration increased. Overall, the nutritional status and depth of the anestrous state was determined to provide possible insight to a mare's response to treatment. Further research was performed to understand the mechanisms of how a combined estradiol-sulpiride treatment induces early follicular growth and ovulation in anovulatory mares. Research on rats

suggests that PRL is responsible for inducing functional LH receptors on granulosa cells and the corpus luteum (Holt et al., 1976; Richards and Williams, 1976; Bjurulf et al., 1994). Oberhaus et al. (2018) used a combined estradiol-sulpiride treatment to assess changes in LH receptor gene expression of early induced ovulatory-sized follicles in mares relative to the first ovulatory-sized follicles occurring naturally in the spring. On January 16, anovulatory mares were administered either 50 mg ECP alone or ECP followed by two 3-g sulpiride injections 5 and 12 d later. Half of each group also received complete follicle ablation before ECP treatment. Follicular fluid was collected via transvaginal aspiration, and blood was harvested to evaluate circulating hormone concentrations. Ablation before treatment did not affect ovarian response. Circulating PRL concentration was increased in ECP-sulpiride treated mares, but did not indicate ovarian response. Plasma LH concentrations were higher in mares that had an ovarian response. Follicular fluid hormone concentrations were not affected by treatment or ablation. In conclusion, ECP-sulpiride early induced follicles had the same steroid production and mRNA for LH receptors as naturally occurring follicles in the spring, and were therefore deemed just as competent as follicles occurring naturally (Oberhaus et al. 2017).

Embryo Transfer

Embryo transfer is an acceptable assisted reproductive technique for use in the horse, though management of mares involved can be challenged by the anestrous period. Embryo transfer allows for foals to be obtained from mares while they are competing, unable to carry a foal because of nonreproductive health or musculoskeletal problems, with reproductive problems; and multiple foals can be produced by the same mare in the

same year (Brinsko et al., 2011). McCue and Squires (2015) determined that satisfactory pregnancy rates after embryo transfer is typically above 70%. Effectiveness of mare synchronization is often the limiting factor for high usage of recipient mares with good pregnancy rates (McCue and Squires, 2015). The costliest component of an embryo transfer program typically is maintaining and managing recipient mares. A reliable, convenient, cost effective, and proven treatment to synchronize recipients, at any reproductive status, with donor mares would be beneficial.

Rationale for Present Experiment

Substantial information is still needed to understand the mechanism by which a mare transitions in and out of cyclicity, and more research would increase the understanding of PRL activity in correspondence with ovarian stimulation after anestrus. The equine reproduction industry would benefit from a more convenient, effective, and safe procedure for inducing earlier cyclicity that is capable of maintaining a pregnancy. Increased PRL concentrations in the mare evidently promotes follicular activity and can lead to ovulation; however, further investigation into the long-term effects of PRL leading to a pregnancy is needed. No previous study was identified in the literature that evaluated the efficacy of a single dose of ECP followed by a single dose of sulpiride one day later to induce early cyclicity on mares that represented varying status within the anestrous season. Therefore, the objectives of the experiment presented herein were to evaluate the effect of ECP and sulpiride treatment on: 1) induction of early cyclicity in anestrous mares, 2) factors that may prevent or contribute to an individual mare's response, 3) establishment of pregnancy earlier in the breeding season, 4) plasma PRL and LH concentrations post-treatment, and 5) follicular activity post-treatment.

CHAPTER III

MATERIALS AND METHODS

An experiment was designed to evaluate the effect of a combined ECP-sulpiride treatment on anestrous mares. This experiment was conducted under the supervision and approval of the West Texas A&M University Institutional Animal Care and Use Committee (Protocol # 2019.03.001).

Experimental Design

Animals

In January to March 2020, 32 mares with a known reproductive history were used in a clinical study to use a combined estradiol-sulpiride treatment to induce cyclicity in anestrous mares. Clinically healthy mares (n = 32) intended for use as recipients in an embryo transfer program, ranging from 7 to 21 yr of age, weighing between 500 to 900 kg, within a body condition score of 5 to 8 (Henneke et al., 1983), and were a mix of stock and draft-type were used in the experiment. Mares were housed at Timber Creek Veterinary Hospital, Canyon, Texas, under natural photoperiod. Mares were housed together randomly across treatments in pens (25 x 25 m) with 10 to 15 horses per pen, and received a diet of alfalfa hay twice daily and provided water ad libitum.

Beginning January 9th, all mares were transrectally palpated and scanned with an ultrasound (SonoSite Edge II with a 10-5 MHz transducer; FUJIFILM SonoSite, Inc.,

Bothell, WA) to assess ovarian activity. Venous blood was collected via jugular venipuncture before the start of the experiment on January 9 and 17 for the determination of ovarian status and possible luteal activity as reflected by progesterone concentrations. Mares were placed randomly in one of four treatment groups based on their ovarian activity and blood progesterone concentrations prior to the start of the trial. Eleven mares were determined to be in deep anestrus and placed in the anestrous group as a result of having follicles less than 10 mm and plasma progesterone concentration less than 0.2 ng/mL. Eight mares were placed in a transitional group that had multiple small follicles less than 15 mm and progesterone concentrations between 0.2 to 13.4 ng/mL. Nine mares were placed in a progesterone group because they were pretreated with 300 mg altrenogest (NexGen Pharmaceuticals, Weatherford, Texas) either on December 27 or 30. All 9 mares in the progesterone group had plasma progesterone concentrations less than 0.2 ng/mL, 7 mares had follicles less than 10 mm, and 2 mares had follicles less than 15 mm. Four mares were randomly selected and placed in a control (C) group and had follicles less than 10 mm and plasma progesterone concentrations less than 0.2 ng/mL.

Treatments

Beginning on d -1, the 28 treated mares (anestrus, n = 11; transitional, n = 8; progesterone, n = 9) were administered 50 mg ECP (BET Pharm, Lexington, KY) intramuscularly. Control mares (n = 4) received an equivalent volume intramuscular injection of saline. On d 0, about 24 h following ECP administration, 3 g sulpiride (Sigma-Aldrich, St. Louis, MO) in 2 mL of sucrose acetate isobutyrate (SAIB; Eastman Chemical Company, Kingsport, TN) was administered intramuscularly to treated mares. The C group received 2 mL SAIB only.

Embryo Transfer

After mares went through at least 2 cycles, embryos of various types (Table A-1) were transferred depending upon when they were synchronized with a donor or targeted date of transfer. Four embryos were frozen within 24 h of the donor flush at -196°C for a minimum of 6 mo, 2 embryos were transferred fresh within 24 h of the donor flush, and 4 embryos were vitrified after somatic cell nuclear transfer and stored for a minimum of 6 mo at -196°C. All mares that received embryos were sedated, and an embryo was transferred transcervically into the uterus with a cuozzo gun (IMV Technologies, L'Aigle, France) between 4 to 8 d after ovulation. Recipient mares were scanned via ultrasound to assess pregnancy status at 13 d post-ovulation and periodically thereafter.

Sample Collection and Analysis

Blood Collection

Blood samples were collected by jugular venipuncture from each mare at 0700 at 0, 3, 6, 12, and 24 h post-sulpiride administration followed daily for 10 d, and every other day for another 32 d. Blood samples were collected with 20-gauge needles into 10-mL evacuated glass tubes containing 158 United States Pharmacopeia units of sodium heparin (Becton, Dickinson & Co., Franklin Lakes, NJ). All blood samples were centrifuged within 30 min of collection at 1321 x *g* for 10 min. Plasma was harvested and stored frozen (-15°C) until the completion of the experiment.

Palpation and Ultrasound

Beginning on d 3, all mares had their ovaries scanned to assess ovarian activity and uterine status. Thereafter, mares were palpated weekly on Monday, Wednesday, and Friday. Once a 35 mm follicle or larger was detected, to follow an industry standard

procedure, 1,500 units hCG (Merck Animal Health Inc, Summit, NJ) was administered to induce ovulation. Once ovulation occurred, mares were allowed a natural diestrous phase and palpated until a subsequent ovulation was detected. Mares were followed by ultrasound for 60 d after sulpiride injection. Observations were recorded for mammary gland development to assess any visual signs of the lactational effect of PRL.

Blood Sample Analysis

The frozen blood plasma samples were transported by personal vehicle in containers that maintained temperatures below 0 °C to Louisiana State University, and analyzed in the Ag Center Animal and Food Sciences Laboratory (Baton Rouge, LA). Samples were assayed for plasma PRL concentrations at h 0, 3, 6, 12, and 24, and d 2 through 10; and LH concentrations at h 0, 3, 6, 12, and 24, and d 2 through 10, and every other day from d 12 through 40. Prolactin and LH plasma concentrations were estimated by radioimmunoassay previously validated for equine plasma (Colborn et al., 1991a). Inter- and intra-assay coefficients of variation, and limits of sensitivity were 7%, 12%, and 0.2 ng/mL for PRL, and 6%, 9%, and 0.2 ng/mL for LH, respectively.

Statistical Analysis

Successful first and second ovulation within the selected time period were coded 1, and failure to ovulate was coded 0. These binomial data were analyzed with the GLIMMIX procedure of SAS (SAS Institute Inc., Cary, NC, 2012). Number of days to a follicle greater than 20 mm, first ovulation, second ovulation, and days from first to second ovulation were analyzed by one-way ANOVA for a completely randomized design via MIXED procedure of SAS. A contrast statement was included to compare the mean of the 3 treated groups to C. Hormonal concentration data from daily and frequent

blood samples were analyzed using the MIXED procedure of SAS with repeated measures. The model for these variables included main effects of treatment and time, and the two-way interaction of treatment and time. The repeated statement was day, and the covariance structure with the lowest Akaike information criterion for each dependent variable was used. A correlation analysis was performed between day of peak LH concentration and the day of ovulation using the CORR procedure of SAS to generate a Pearson correlation coefficient. Plasma mean PRL and LH concentrations were tested for normal distribution using the Shapiro–Wilk test within PROC UNIVARIATE to determine if dependent variables were normally distributed. Nonparametric data were natural log-transformed and again tested for normal distribution. If natural log-transformation improved normality, the log-transformed data were statistically analyzed, and back-transformed means were subsequently generated and reported. Statistical significance was established for main effects of treatment and day, and the interaction of treatment and day if the resulting *P*-value was less than or equal to 0.05. Statistical tendency was considered if the resulting *P*-value was less than or equal to 0.10 and greater than 0.05. Differences of Least squares means were determined using the PDIFF option in SAS with $\propto$ equal to or less than 0.05 used to declare significance.

CHAPTER IV

RESULTS AND DISCUSSION

The number and percentage of mares by treatment with first ovulation within 28 d, and second ovulation within 60 d after ECP and sulpiride, or carrier injection is shown in Table 1. There was an effect of treatment on the number of mares that had a first ovulation ($P < 0.001$) within 28 d. Of the 28 mares that were treated with ECP and sulpiride, 23 (82.1%) ovulated within 22 d in comparison to 0 of 4 (0%) of C. There was an effect of treatment ($P < 0.001$) of the percentage of mares with first ovulation within 28 d between the contrast of the percentage mean of the 3 ECP-sulpiride treated groups (transitional, anestrous, and progesterone; $\bar{x} = 82.8\%$) and the C ($\bar{x} = 0\%$) group. Mares in the transitional group tended to ovulate at a higher percentage than the progesterone group ($P = 0.067$). There was no statistical difference of the percentage of mares with a first ovulation between the transitional and anestrous groups ($P = 0.28$), or the anestrous and progesterone groups ($P = 0.36$). The transitional group had 8 of 8 (100%) mares achieve a first ovulation. Oberhaus et al. (2017) reported that the likelihood of a seasonally anovulatory mare responding to a similar treatment protocol as to the ECP-sulpiride protocol used in the current study, was inversely correlated to depth of anestrus. Similarly, Panzani et al. (2011) concluded that using a sulpiride treatment on mares with

follicles greater than 25 mm in diameter reduced the interval between the first day of treatment and ovulation compared to mares in deep anestrus.

There was an effect of treatment on the number of mares that had a second ovulation ($P = 0.018$). In the current study, after mares had a natural diestrous period, 15 of the 23 (65.2%) mares across all 3 of the ECP-sulpirde treated groups that had first ovulations had a second ovulation and C had 0% second ovulation. The transitional group tended ($P = 0.053$) to have a higher percentage (87.5%) of mares with second ovulation than the anestrous group (45.5%), and had higher percentages than the progesterone ($P = 0.019$, 33.3%) and control ($P = 0.004$, 0.0%) groups. The anestrous group tended ($P = 0.093$) to have a second ovulation at a higher percentage than the control. There were no statistical differences between the anestrous and progesterone ($P = 0.55$) groups, and the progesterone and control ($P = 0.23$) groups. The 8 ECP-sulpiride treated mares that did not have a second ovulation after having a first appeared to return to an anestrous or transitional state by having a longer than normal interovulatory period. Zero C mares had a second ovulation within 60 d of carrier injection.

All mares within all 4 treatment groups, including C, developed follicles greater than 20 mm within 22 d from sulpiride or carrier injection, but all mares did not continue to develop follicles to ovulation. For this study, 28 d was predetermined to be the length of time allowed for a mare to have first ovulation in response to ECP and sulpiride. Previous research support that if a mare had not responded and ovulated within 28 d of the treatment, she was most likely not going to respond at all (Oberhaus et al., 2017). In the present study, all ECP-sulpiride treated mares that had an ovulation, actually ovulated within 22 d.

The mean number of days by treatment to first follicle greater than 20 mm, first and second ovulation, and days from first to second ovulation after ECP and sulpiride treatment, or carrier injection are presented in Table 2. There was an effect of treatment ($P < 0.001$) on number of days to first ovulation. The mean of the number of days to first ovulation of the 3 ECP-sulpirde treated groups (transitional, anestrous, and progesterone; $\overline{x} = 12.67$) was 32 ± 5 d earlier ($P < 0.001$) than C ($\overline{x} = 45.0$). The transitional group ovulated sooner at 9.1 d ($P = 0.05$) than the anestrous group (14.8 d), and tended ($P = 0.07$) to ovulate sooner than progesterone group (14.0 d). Oberhaus et al. (2018) reported a similar mean day advancement of ovulation between ECP-sulpiride treated mares compared to C mares of 35 ± 10 d.

While mare's individual response varied, differences in protocol between previous experiments seemed to have an effect on the outcome of the mare's response as well. Previous protocols have utilized multiple injections of ECP or estradiol and/or sulpiride. In 2002, Donadeu and Thompson administered sulpiride daily for 32 d (1mg/kg of BW in vegetable shortening and 250 mg sulpiride/mL vegetable shortening). In 2006, Kelley et al. administered 250 mg sulpiride in vegetable shortening daily for 45 d or until ovulation. Mari et al. (2009) treated mares daily for 25 d with 1 mg/kg of BW of sulpiride. Oberhaus et al. (2017) used a single injection of sulpiride in 5 mL of vegetable shortening in an effort to simplify the previous protocol. In previous research, ovarian success was defined by ovulation within 28 d of sulpiride. The authors observed that as the number of injections was reduced, the success rates decreased as well (Oberhaus et.al., 2017). Kelley et al. (2006) observed a 89% success rate of mares ovulating early after estradiol benzoate injection every other day with sulpiride administered daily 11 d

after the start of estradiol treatment. In contrast, Oberhaus et.al. (2017) observed a 39% success rate with a single injection of ECP and a single injection of sulpiride. In the present study, 82% of treated mares ovulated after a single injection of ECP and a single injection of Sulpiride. The present study utilized SAIB as the carrier for sulpiride instead of vegetable shortening that was previously used, suggesting that the carrier could impact the response. Arana-Valencia et al. (2018) conducted multiple experiments to compare factors that affect the PRL response to sulpiride, including enantiomer composition, oral administration, and various carrier formulations, with the goal of a single-injection protocol with high efficacy with an extended period of stimulation on plasma PRL concentrations. Because SAIB was not available, SucroMate was used as one of the carriers for sulpiride in the experiment as Sucromate is a GnRH analog in SAIB. Prolactin response to 3 g of sulpiride in vegetable shortening and 3 g sulpiride in 5 mL of SucroMate or just SurcoMate (control) was compared in ECP-primed geldings. Prolactin responses persisted at least 10 d and control geldings had no PRL response to SucroMate. Prolactin concentrations in geldings receiving sulpiride in vegetable shortening were generally lower than those in geldings receiving sulpiride in SucroMate. The planned differences in protocol of the present study from other studies, with the unique chemical composition of ECP followed by administration of sulpiride 24 h later appeared to result in positive outcomes.

The differences in climate and length of day that is associated with latitude from the present research to previous locales should be noted. Temperature could potentially influence a mare's response to ECP and sulpiride. Previous research at Louisiana State University, which is located at 30.471165 latitude and in a humid subtropical climate

(Kottek et al., 2006). The present study was performed at 35.199165 latitude and a temperate semi-arid climate in the Texas Panhandle (Kottek et al., 2006). The average temperature for the months of January and February are 5.1 and 3.7°C for Amarillo, TX and 13.6 and 13.9°C for Baton Rouge, LA, respectively (National Oceanic and Atmospheric Administration Online Weather Data, 2006). Further research is needed to further understand how temperature and climate may affect a mare's anestrous period and the mechanism of transitioning to cyclicity.

Mean plasma PRL concentrations by treatment for 10 d and 72 h after ECP and sulpiride, or carrier injection are shown in Fig. 1 and 2, respectively. There was an effect of treatment ($P < 0.001$) and a treatment-by-day interaction ($P < 0.001$) on mean plasma PRL concentrations. In the present study, mares treated with ECP and sulpiride treatment increased PRL concentrations by 9 to 16 ng/mL over the concentrations in C mares at 3 h after sulpiride administration. Difference in PRL concentrations between ECP-sulpiride treated groups and C persisted through d 9.

Oberhaus et al. (2017) observed that a mare's individual response to ECP and sulpiride can vary from season to season; while some mares respond similarly each season, some mares never respond, and other mares will respond one season, but not the next season. Research has been conducted to evaluate factors that may contribute or prevent a mare's response to treatment. In the present study, PRL concentrations was one factor that was evaluated. Mean plasma concentrations for treated mares that ovulated within 28 d (n = 23) and treated mares that did not ovulate within 28 d (n = 5) are shown in Fig. 3. There was no difference ($P = 0.390$) in mean plasma PRL concentrations for 10 d after sulpiride injection between treated mares that ovulated within 28 d and treated

mares that did not ovulate. Similarly, Oberhaus et al. (2018) reported no difference in PRL response to sulpiride between mares that had an early ovulatory-sized follicle (35 mm) and those that did not develop a follicle to 35 mm.

Mean plasma LH concentrations by treatment during the first 40 d of treatment are presented in Fig. 4. Although there was no effect of treatment ($P = 0.17$) on plasma LH concentration observed, there was an effect of the interaction of treatment and day ($P = 0.021$). Particularly, ECP-sulpiride treated groups tended to have higher LH concentrations on d 3 through 20 ($P < 0.10$) compared to C. Peak LH concentrations for individual mares had a strong linear correlation with day of ovulation ($R = 0.87$; $P < 0.001$).

Plasma LH tended to be higher ($P < 0.08$; Fig. 5) in treated mares that had a first ovulation between d 14 to 22 than mares that did not have a first ovulation. Similarly, Oberhaus et al. (2017) did not observe a difference in LH concentrations until d 10 at one farm and d 14 at another farm. In 2018, Oberhaus et al. reported a difference in plasma LH concentrations in responding mares and non-responding mares on d 8 and 12 after sulpiride injection. In order to have an ovulation there appears to be a threshold of PRL and LH concentrations that must be attained. Mitcham et al. (2013) administered injections of ECP alone, with no dopamine antagonist to stimulate PRL, and failed to stimulate early ovulation. Oberhaus et al. (2017) observed that PRL response to sulpiride was large enough to reach the threshold required for ovulation in most cases. Mares that did not ovulate on 1 farm had smaller LH responses and must not have reached the LH threshold required for ovulation. The authors concluded that failure of either PRL or LH responses resulted in failure to stimulate an early ovulation. Plasma concentrations for

LH and PRL in the present study were similar to the results reported by Oberhaus et al. (2018).

The mean age of mares was 16.0, 14.8, 13.9, and 12.9 yr for the control, anestrous, transitional, and progesterone groups, respectively. There was no differences of age across treatment groups ($P = 0.61$). Of the treated mares, age did not appear to have an effect on determining if a mare achieved an ovulation or not ($P = 0.20$). The mean age of mares that ovulated within 28 d was 14.2 yr, and the mean age of mares that did not ovulate was 11.8 yr. Another factor that has been reported to effect response to ECP and sulpiride is body condition and nutritional status (Oberhaus et al., 2017). All mares in the study were between 5 to 8 in body condition score (Henneke et al., 1983), and therefore, no mares were excessively over or underweight.

Data for embryos used for transfer and mare's pregnancy status at 60 d post-transfer are presented in Table A-1. Ten embryos were transferred in the present study after at least a second ovulation. The percentage of confirmed pregnancies after embryo transfer into mares treated with ECP-sulpiride was 70% (7 of 10). Mari et al. (2009) and Panzani et al. (2011) attempted to estimate fertility using artificial insemination after administration of sulpiride or domperidone to induce ovulation in deep anestrous mares. Unlike previous research reports, mares in the present study were assessed for pregnancy after embryo transfer. Embryos were not transferred into the recipient mares in this study until at least the second or later ovulation to align with preferred foaling dates. Transferring embryos to mares after first ovulation had the possibility of resulting in a foal born in December. Thus, mare's ovarian activity were followed to at least the second ovulation before an embryo was transferred. Of the 15 mares that had second ovulations,

10 of them received transferred embryos. The other 5 mares were not used due to reasons outside of the experiment. Oliveira Neto et al. (2018) compared recipient mares at various stages of their cycle and used a estradiol-progesterone treatment to synchronize them to donors, then assed pregnancy rates from the embryo transfers. Prior to the sulpiride treatment, the estradiol-progesterone treatment described in this research was the common practice of Timber Creek Veterinary Hospital. Data from this study suggest that mares in anestrous, spring transition, early estrous, estrous, diestrous and early diestrous stages of the cycling year or season could respond to a estradiol-progesterone treatment. In the Oliveira Neto et al. (2018) study, the anestrous group (n = 20) had a pregnancy rate of 90% up to 120 d. In contrast, the pregnancy rate for the current study of embryo transfer into ECP-sulpiride treated mares was 70%. The success of an embryo transfer program could be the result of various factors such as management and fertility of dams and sires, experience of the professional(s) collecting and transferring embryos, and quality and management of recipient mares (McCue and Squires, 2015; Oliveira Neto et al., 2018). The current protocol was advantageous as only 2 injections were used instead of multiple injections over multiple days. The present protocol in comparison with that of Oliveira Neto et al. (2018) reduces labor involved with treatment and potential cost. Similar pregnancy rates were reported following a dopamine antagonist treatment as Mari et al. (2009), who reported a pregnancy rate of 40% (4 of 10) and 70% (7 of 10) for mares treated with sulpiride or domperidone, respectively. Panzani et al. (2011) reported a 63.6% pregnancy rate (14 of 22) in mares treated with sulpiride and inseminated after first ovulation.

CHAPTER V

CONCLUSION AND IMPLICATION

In conclusion, a combined treatment of a single injection of ECP followed one day later with a single injection of sulpiride increased plasma PRL concentrations and hastened the days to first ovulation. Of the 82% of treated mares that ovulated within 22 d of treatment, 65% continued cycling to a second ovulation within 43 d. An increase in PRL concentrations were stimulated in ECP-sulpiride treated mares for 9 d. Age appeared to have no effect on whether or not a mare responded to the ECP-sulpiride treatment. In agreement with previous research, the depth of anestrus appeared to have an effect on ovulation response to ECP and sulpiride treatment. Transitional mares had the highest ovulation rate and shortest interval of days from treatment to first ovulation. Lastly, 70% of treated mares maintained a pregnancy after embryo transfer suggesting that subsequent cycles following ECP and sulpiride treatment appeared to be normal and capable of sustaining a pregnancy. The unique chemical composition of ECP appears to enhance the effect of sulpiride. At present, sulpiride is a compounded drug that is not commercially available, but for use only by a veterinarian. Overall, the treatment procedure in this study appeared to be an effective approach for mares in a variety of management conditions, including being housed outside, to hasten normal cyclicity earlier in the year.

LITERATURE CITED

Alexander, S. L., and C. H. G. Irvine. 1996. GnRH secretion in the mare. Anim. Reprod. Sci., 42, 173-180.

Alexander, S. L., C. H. G. Irvine, and M. J. Evans. 2004. Inter-relationships between the secretory dynamics of thyrotrophin-releasing hormone, thyrotrophin and prolactin in periovulatory mares: effect of hypothyroidism. J. Neuroendocrinology. 16, 906-915.

Arana-Valencia, N., D. L. Thompson Jr, and E. L. Oberhaus. 2018. Dopaminergic and antidopaminergic effects on heart rate in healthy horses when challenged with brief 2-minute exercise bouts. J. Equine Vet. Sci. 71, 120.

Aurich, C. 2011. Reproductive cycles of horses. Anim. Reprod. Sci. 124, 220.

Aurich, C., P. F. Daels, B. A. Ball, and J. E. Aurich. 1995. Effects of gonadal steroids on the opioid regulation of LH and prolactin release in ovariectomized pony mares. J. Endocrinology. 147, 195.

Ben-Jonathan, N., and R. Hnasko. 2001. Dopamine as a prolactin inhibitor. Endocr. Rev. 22, 724.

Besognet, B., B. S. Hansen, and P.F. Daels. 1996. Dopaminergic regulation of gonadotrophin secretion in seasonally anoestrous mares. J. Reprod. Fertil. 108, 55.

Besognet, B., B. S. Hansen, and P. F. Daels. 1997. Induction of reproductive function in anestrous mares using a dopamine antagonist. Theriogenology. 47, 467.

Bjurulf, E., G. Selstam, and J. I. Olofsson. 1994. Increased LH receptor mRNA and extended corpus luteum function induced by prolactin and indomethacin treatment in vivo in hysterectomized pseudopregnant rats. J. Reprod. Fertil. 102, 139-45.

Brendemuehl, J. P., and D. L. Cross. 2000. Influence of the dopamine antagonist domperidone on the vernal transition in seasonally anoestrous mares. J. Reprod. Fertil. 185.

Brinsko, S. P., T. L. Blanchard, D. D. Varner, J. Schumacher, C. C. Love, K. Hinrichs, and D. Hartman. 2011. Manual of Equine Reproduction. Equine Reproduction.

Burns, J. P., D. L. Thompson Jr., W. A. Storer, R. Gilley, C. Morrow, J. Abraham, and R. H. Douglas. 2006. New pharmacological treatments for equine reproductive management. In: BioRelease Technologies 1st Annual Review. Lexington, KY.

Clayton, R. N. 1989. Gonadotrophin-releasing hormone: its actions and receptors. J. Endocrinology. 120(1), 11-19.

Colborn, D. R., D. L. Thompson Jr., M. S. Rahmanian, and T. L. Roth. 1991a. Plasma concentrations of cortisol, prolactin, luteinizing hormone, and follicle-stimulating hormone in stallions after physical exercise and injection of secretagogue before and after sulpiride treatment in winter. J. Anim. Sci. 69, 3724.

Colborn, D. R., D. L. Thompson Jr., T. L. Roth, J. S. Capehart, and K. L. White. 1991b. Responses of cortisol and prolactin to sexual excitement and stress in stallions and geldings. J. Anim. Sci. 69, 2556.

Creese, I., D. R. Sibley, M. W. Hamblin, and S. E. Leff. 1983. The classification of dopamine receptors: relationship to radioligand binding. Annu. Rev. Neurosci. 6, 43-71.

Donadeu, F. X., and D. L. Thompson Jr. 2002. Administration of sulpiride to anovulatory mares in winter: effects on prolactin and gonadotropin concentrations, ovarian activity, ovulation, and hair shedding. Theriogenology. 57, 963.

Duchamp G., and P. F. Daels. 2002. Combined effect of sulpiride and light treatment on the onset of cyclicity in anestrous mares. Theriogenology. 58, 599.

Fitzgerald, B. P., and C. J. McManus. 2000. Photoperiodic versus metabolic signals as determinants of seasonal anestrus in the mare. Biol. Reprod. 63, 335.

Freeman, M. E., B. Kanyicska, A. Lerant, and G. Nagy. 2000. Prolactin: structure, function, and regulation of secretion. Physiol. Rev. 80, 1523-631.

Ginther, O. J. 1979. Reproductive biology of the mare - basic and applied aspects. xv 413 pp. Department of Veterinary Science, University of Wisconsin. Madison, WI.

Hadley, M. E. 2000. Endocrinology. Fifth Edition ed. Prentice Hall, Inc., Upper Saddle River, NJ.

Henneke, D. R., G. D. Potter, J. L. Kreider, B. F. Yeates. 1983. Relationship between condition score, physical measurements and body fat percentage for mares. J. Equine Vet. Sci. 15, 371-6.

Holt, J. A., J. S. Richards, A. R. Midgley Jr., and L. E. Reichert Jr. 1976. Effect of prolactin on LH receptor in rat luteal cells. Endocrinology. 98, 1005-13.

Irvine, C. H., and S. L. Alexander. 1993. Secretory patterns and rates of gonadotropin-releasing hormone, follicle-stimulating hormone, and luteinizing hormone revealed by intensive sampling of pituitary venous blood in the luteal phase mare. Endocrinology. 132, 212.

Irvine, C. H., S. L. Alexander, and A. O. McKinnon. 2000. Reproductive hormone profiles in mares during the autumn transition as determined by collection of jugular blood at 6 h intervals throughout ovulatory and anovulatory cycles. J Reprod. Fertil. 118, 101-9.

Johnson, A. L., and S. E. Becker. 1987. Effects of physiologic and pharmacologic agents on serum prolactin concentrations in the nonpregnant mare. J. Anim. Sci. 65, 1292.

Kebabian, J. W., and D. B. Calne. 1979. Multiple receptors for dopamine. Nature. 277, 93-6.

Keeler, J. F., D. O. Pretsell, and T. W. Robbins. 2014. Functional implications of dopamine D1 vs. D2 receptors: A 'prepare and select' model of the striatal direct vs. indirect pathways. Neurosci. 282, 156-175.

Kelley, K. K., D. L. Thompson Jr., W. A. Storer, P. B. Mitcham, R. M. Gilley, and P.J. Burns. 2006. Estradiol interactions with dopamine antagonists in mares: prolactin secretion and reproductive traits. J. Equine Vet. Sci. 26, 517.

Kottek, M., J. Grieser, C. Beck, B. Rudolf, and F. Rubel. 2006. World map of the koppen-geiger climate classification updated. Meteorol. Z., 15, 259-263.

Langan, E. A., E. Hinde, and R. Paus. 2018. Prolactin as a candidate sebotrop(h)ic hormone. Exp. Dermatol. 27, 729-736.

Mari, G., M. Morganti, B. Merlo, C. Castagnetti, F. Parmeggiani, N. Govoni, G. Galeati, and C. Tamanini. 2009. Administration of sulpiride or domperidone for advancing the first ovulation in deep anestrous mares. Theriogenology. 71, 959.

McCue, P. M., and E. L. Squires. 2015. Equine embryo transfer. Teton NewMedia, Print. Jackson, WY.

Melrose, P. A., R. F. Walker, and R. H. Douglas. 1990. Dopamine in the cerebrospinal fluid of prepubertal and adult horses. Brain, Behavior and Evolution. 35, 98-106.

Mitcham, P. B., D. L. Thompson, P. J. Burns, R. M. Gilley, and R. C. Hebert. 2013. Development of an estradiol-dopamine antagonist protocol for inducing ovulation in seasonally anovulatory mares. J. Equine Vet. Sci. 33, 375-376.

Mitcham, P. B., D. L. Thompson Jr., T. T. Thompson, S. D. Bennett, P. J. Burns, and T. J. Caltabilota. 2010. Stimulation of ovulation in seasonally anovulatory and vernal transitional mares with estradiol and domperidone: dose and combination studies. J. Equine Vet. Sci. 30, 244.

Munemura, M., T. E. Cote, K. Tsuruta, R. L. Eskay, and J. W. Kebabian. 1980. The dopamine receptor in the intermediate lobe of the rat pituitary gland: pharmacological characterization. Endocrinology. 107, 1676-1683.

National Oceanic and Atmospheric Administration Online Weather Data (nowdata): Interactive Data Query System: Public Fact Sheet. 2006, Washington, D.C.: National Oceanic and Atmospheric Administration, Internet resource.

Nequin, L. G., S. S. King, A. L. Johnson, G. M. Gow, and G. M. Ferreira-Dias. 1993. Prolactin may play a role in stimulating the equine ovary during the spring reproductive transition. J. Equine Vet. Sci. 13, 631-635.

Niswender, K. D., P. M. McCue, and R. L. Squires. 2004. Effect of purified equine follicle-stimulating hormone on follicular development and ovulation in transitional mares. J. Equine Vet. Sci. 24, 37-39.

Oberhaus, E. L., K. L. Jones, and S. S. King. 2015. Immunohistochemical localization of prolactin receptors within the equine ovary. J. Equine Vet. Sci. 35, 7.

Oberhaus, E. L., and D. Paccamonti. 2013. Review of management of anestrus and transitional mares. Am. Assoc. Equine Practitioners. Proc. 59, 329.

Oberhaus, E. L., D. L. Thompson Jr., B. A. Foster, and C. R. Pinto. 2018. Effects of combined estradiol-sulpiride treatment and follicle ablation on vernal transition in mares: evaluation of plasma and follicular fluid hormones and luteinizing hormone receptor gene expression. J. Equine Vet. Sci. 64, 69.

Oberhaus, E. L., D. L. Thompson Jr., and C. K. Pham. 2017. Factors affecting the ovarian response to a combined estradiol-sulpiride treatment in seasonally anovulatory mares. J. Equine Vet. Sci. 50, 129.

O'Connor, S. E., and R. A. Brown. 1982. The pharmacology of sulpiride—A dopamine receptor antagonist. General Pharmacology: The Vascular System. 13, 185-193.

Oliveira Neto, I. V., I. F. Canisso, L. G. Segabinazzi, C. P. F. Dell'Aqua, M. A. Alvarenga, F. O. Papa, and J. A. Dell'Aqua Jr. 2018. Synchronization of cyclic and acyclic embryo recipient mares with donor mares. Anim. Reprod. Sci. 190, 1.

Panzani, D., I. Zicchino, A. Taras, P. Marmorini, A. Crisci, A. Rota, and F. Camillo. 2011. Clinical use of dopamine antagonist sulpiride to advance first ovulation in transitional mares. Theriogenology. 75, 138.

Rahmanian, M. S., D. L. Thompson Jr., and P. A. Melrose. 1997. Immunocytochemical localization of prolactin and growth hormone in the equine pituitary. J. Anim. Sci. 75, 3010.

Richards, J. S., and J. J. Williams. 1976. Luteal cell receptor content for prolactin (PRL) and luteinizing hormone (LH): regulation by LH and PRL. Endocrinology. 99, 1571-81.

Schauer, S. N., C. Briant, M. Ottogalli, C. Decourt, I. G. Handel, and F. X. Donadeu. 2011. Supplementation of equine early spring transitional follicles with luteinizing hormone stimulates follicle growth but does not restore steroidogenic activity. Theriogenology. 75, 1076.

Squires, E. L. 2008. Hormonal manipulation of the mare: a review. J. Equine Vet. Sci. 28, 627.

Thomas, G. B., J. T. Cummins, L. Cavanagh, and I. J. Clarke. 1986. Transient increase in prolactin secretion following hypothalamo-pituitary disconnection in ewes during anoestrus and the breeding season. J. Endocrinology. 111, 425-31.

Thompson, D. L., Jr., F. Garza, Jr., R. L. St. George, M. H. Rabb, B. E. Barry, and D. D. French. 1991. Relationships among LH, FSH and prolactin secretion, storage and response to secretagogue and hypothalamic GnRH content in ovariectomized pony mares administered testosterone, dihydrotestosterone, estradiol,

progesterone, dexamethasone or follicular fluid. Domestic Anim. Endocrinology. 8, 189-199.

Thompson, D. L., Jr., R. Hoffman, and C. L. DePew. 1997. Prolactin administration to seasonally anestrous mares: reproductive, metabolic, and hair-shedding responses. J. Anim. Sci. 75, 1092.

Thompson, D. L., Jr., L. Johnson, R. L. George, and F. Garza Jr. 1986a. Concentrations of prolactin, luteinizing hormone and follicle stimulating hormone in pituitary and serum of horses: effect of sex, season, and reproductive state. J. Anim. Sci. 63, 854.

Thompson, D. L., Jr., P. B. Mitcham, M. L. Runles, P.J. Burns, and R. M. Gilley. 2008. Prolactin and gonadotropin responses in geldings to injections of estradiol benzoate in oil, estradiol benzoate in biodegradable microspheres, and estradiol cypionate. J. Equine Vet. Sci. 28, 232.

Thompson, D. L., Jr., and E. L. Oberhaus. 2015. Prolactin in the horse: historical perspective, actions and reactions, and its role in reproduction. J. Equine Vet. Sci. 35, 343.

Thompson, D. L., Jr., J. J. Wiest, and T. M. Nett. 1986. Measurement of equine prolactin with an equine-canine radioimmunoassay: seasonal effects on the prolactin response to thyrotropin releasing hormone. Domestic Anim. Endocrinology. 3, 247.

Thomson, C. H., D. L. Thompson Jr., L. A. Kincaid, and M. R. Nadal. 1996. Prolactin involvement with the increase in seminal volume after sexual stimulation in stallions. J. Anim. Sci. 74, 2468.

Van Niekerk, C. H., R. I. Coubrough, and H. W. Doms. 1973. Progesterone treatment of mares with abnormal oestrous cycles early in the breeding season. J. S. Afr. Vet. Med. Assoc. 44, 37-45.

Webel, S. K., and E. L. Squires. 1982. Control of the oestrous cycle in mares with altrenogest. J. Reprod. Fertil. Suppl. 32, 193-198.

Table 1. The effect of treatment on the number and percentage of mares by treatment with first ovulation ($P < 0.001$) within 28 d, and second ovulation ($P = 0.018$) within 60 d after ECP and sulpiride, or carrier injection.

	Treatment Groups				
	Control	Anestrous	Transitional	Progesterone	*P*-value
Total number of mares	4	11	8	9	
Number of mares with first ovulation	0	9	8	6	
Percentage of mares with first ovulation	0^{a}	81.8^{b}	100^{b}	66.7^{b}	0.001
Number of mares with second ovulation	0	5	7	3	
Percentage of mares with second ovulation	0^{a}	45.5^{ab}	87.5^{b}	33.3^{a}	0.018

^{a,b}Means with differing superscripts differ ($P \leq 0.05$).

Treatment:

Control – Four mares with no follicular activity treated with carrier.

Anestrous – Eleven mares with no follicular activity treated with ECP and Sulpiride.

Transitional – Eight mares with multiple small follicles, but no luteal tissue treated with ECP and sulpiride.

Progesterone – Nine non-cycling mares with no luteal tissue pretreated with altrenogest and then treated with ECP and sulpiride.

Table 2. The effect of treatment on the mean number of days to first follicle greater than 20 mm, first and second ovulation, and days from first to second ovulation after ECP and sulpiride, or carrier injection.

	Treatment Groups					
	Control	Anestrous	Transitional	Progesterone	SEM[1]	*P*-value
Mean days to follicle greater than 20 mm	8.7	7.4	4.8	8.7	2.11	0.162
Mean days to first ovulation[2]	45.0[a]	14.8[b]	9.1[c]	14.0[b]	4.15	0.001
Mean days to second ovulation	> 60[a]	38.4[b]	31.7[b]	32.3[b]	8.41	0.045
Mean days from first to second ovulation	> 60[a]	20.8[b]	22.7[b]	18.7[b]	3.80	0.001

[1]Pooled (largest) SE of LS means.

[2]Two of 4 control mares ovulated after 28 d.

[a,b,c]Means with differing superscripts differ ($P \leq 0.05$).

Treatment:

Control – Four mares with no follicular activity treated with carrier.

Anestrous – Eleven mares with no follicular activity treated with ECP and Sulpiride.

Transitional – Eight mares with multiple small follicles, but no luteal tissue treated with ECP and sulpiride.

Progesterone – Nine non-cycling mares with no luteal tissue pretreated with altrenogest and then treated with ECP and sulpiride.

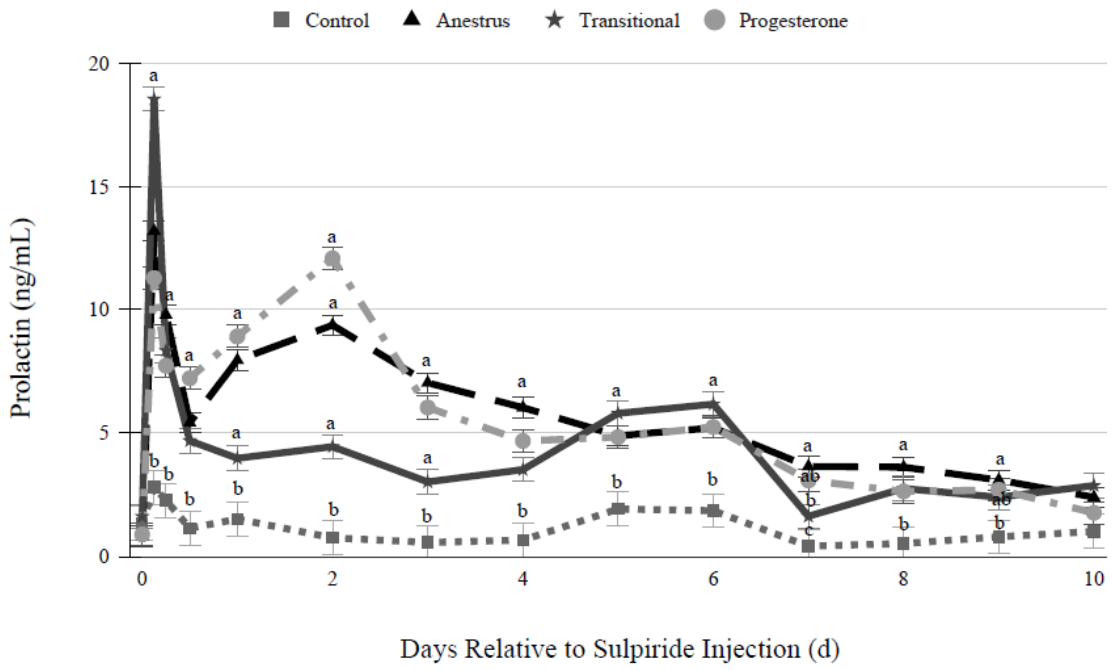

Figure 1. Mean plasma prolactin concentrations by treatment after ECP and sulpiride, or carrier injection for 10 d.

Effect of treatment, $P < 0.001$.
Effect of the interaction of treatment x day, $P < 0.001$.
[a,b,c]Means with differing superscripts differ ($P \leq 0.05$).

Treatment:
Control – Four mares with no follicular activity treated with carrier.
Anestrous – Eleven mares with no follicular activity treated with ECP and Sulpiride.
Transitional – Eight mares with multiple small follicles, but no luteal tissue treated with ECP and sulpiride.
Progesterone – Nine non-cycling mares with no luteal tissue pretreated with altrenogest and then treated with ECP and sulpiride.

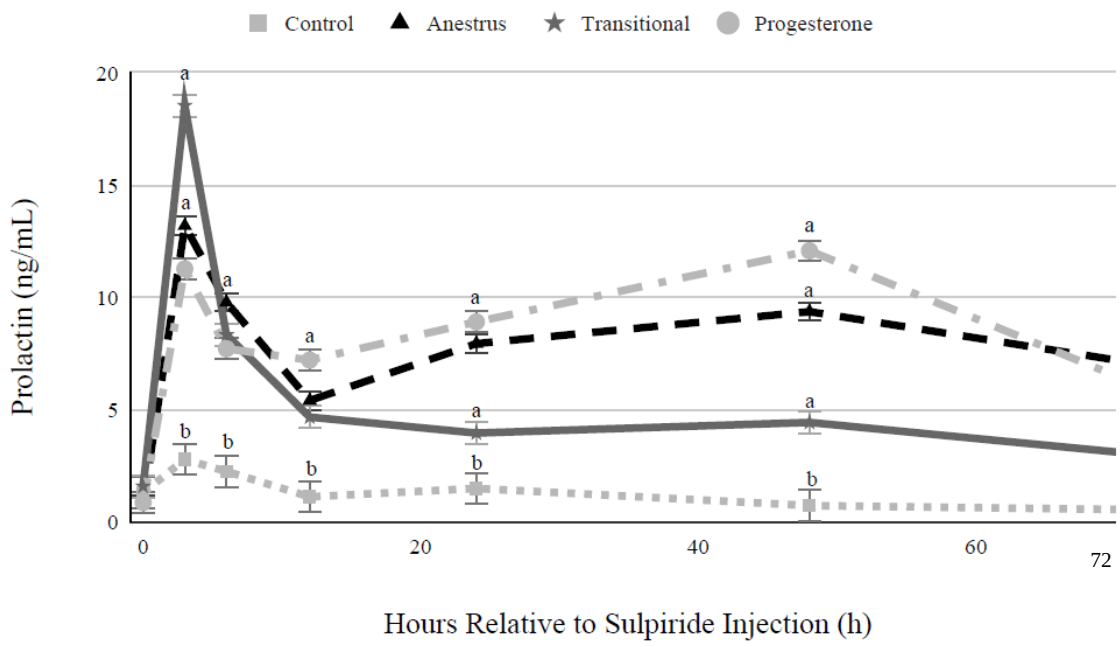

Figure 2. Mean plasma prolactin concentrations by treatment after ECP and sulpiride, or carrier injection for 72 h.

Effect of treatment, $P < 0.001$.
Effect of the interaction of treatment x day, $P < 0.001$.
[a,b,c]Means with differing superscripts differ ($P \leq 0.05$).

Treatment:
Control – Four mares with no follicular activity treated with carrier.
Anestrous – Eleven mares with no follicular activity treated with ECP and Sulpiride.
Transitional – Eight mares with multiple small follicles, but no luteal tissue treated with ECP and sulpiride.
Progesterone – Nine non-cycling mares with no luteal tissue pretreated with altrenogest and then treated with ECP and sulpiride.

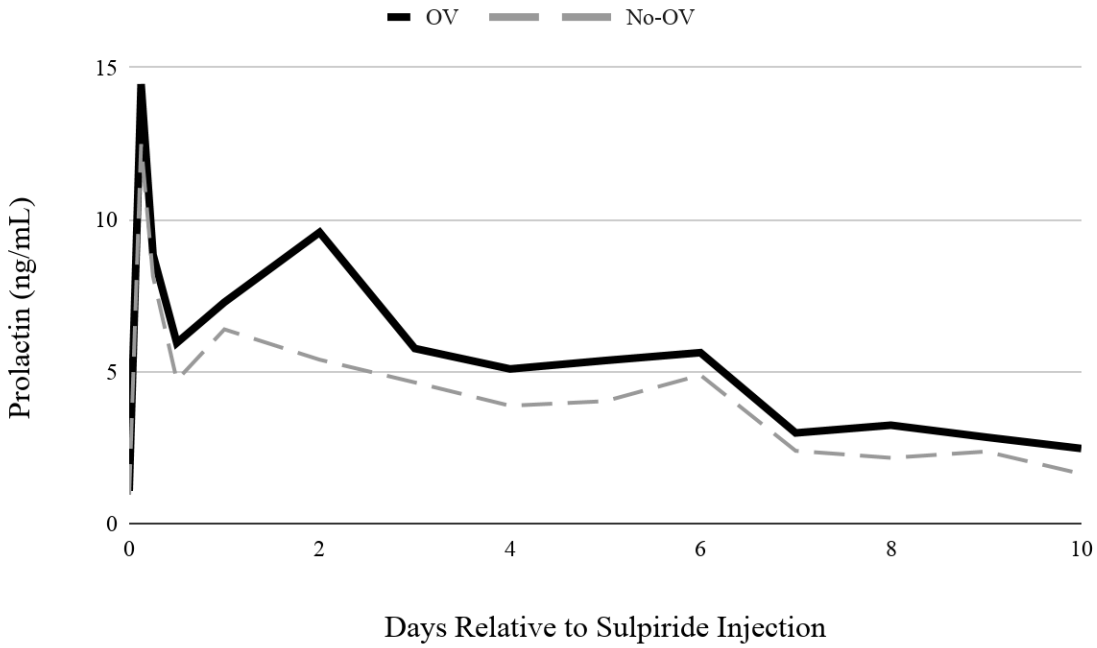

Figure 3. Mean plasma prolactin concentrations for treated mares that ovulated (OV) compared to mares that did not ovulate (No-OV) for 28 d after ECP and sulpiride injection.

OV, n = 23.
No-OV, n = 5.
Effect of the interaction of OV response x day, $P = 0.976$.
Effect of OV response, $P = 0.390$.
Effect of day, $P = 0.001$.

Treated mares: treated with ECP and sulpiride (Anestrous, Transitional, Progesterone groups)

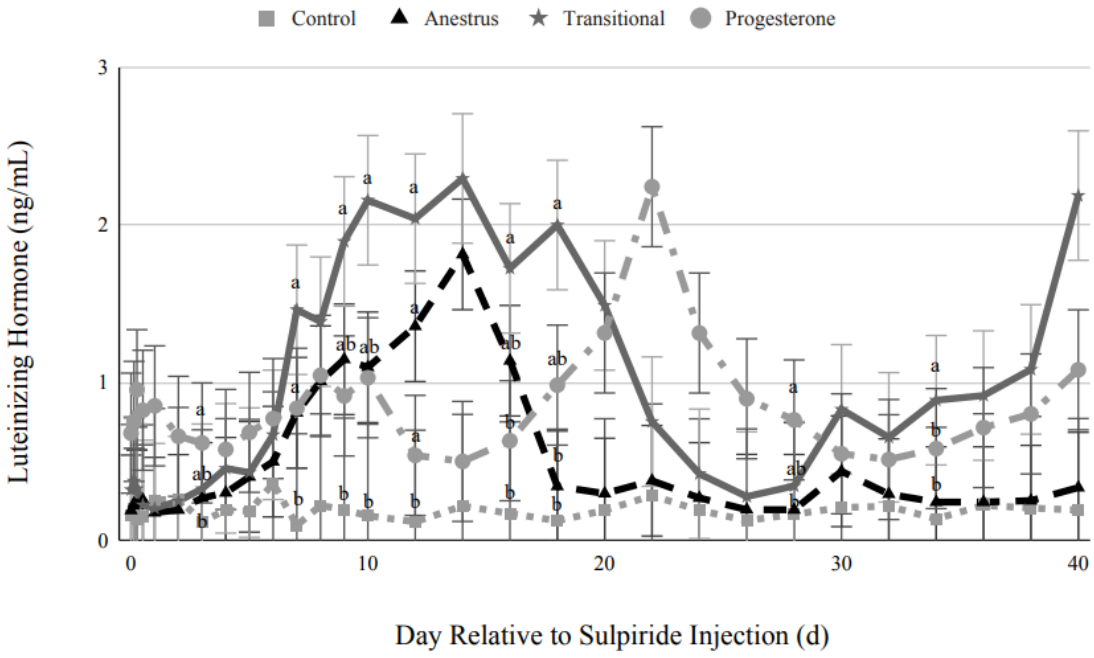

Figure 4. Mean plasma LH concentrations by treatment after ECP and sulpiride, or carrier injection for 40 d.

Effect of the interaction of treatment x day, P = 0.021.

Treatment:
Control – Four mares with no follicular activity treated with carrier.
Anestrous – Eleven mares with no follicular activity treated with ECP and Sulpiride.
Transitional – Eight mares with multiple small follicles, but no luteal tissue treated with ECP and sulpiride.
Progesterone – Nine non-cycling mares with no luteal tissue pretreated with altrenogest and then treated with ECP and sulpiride.

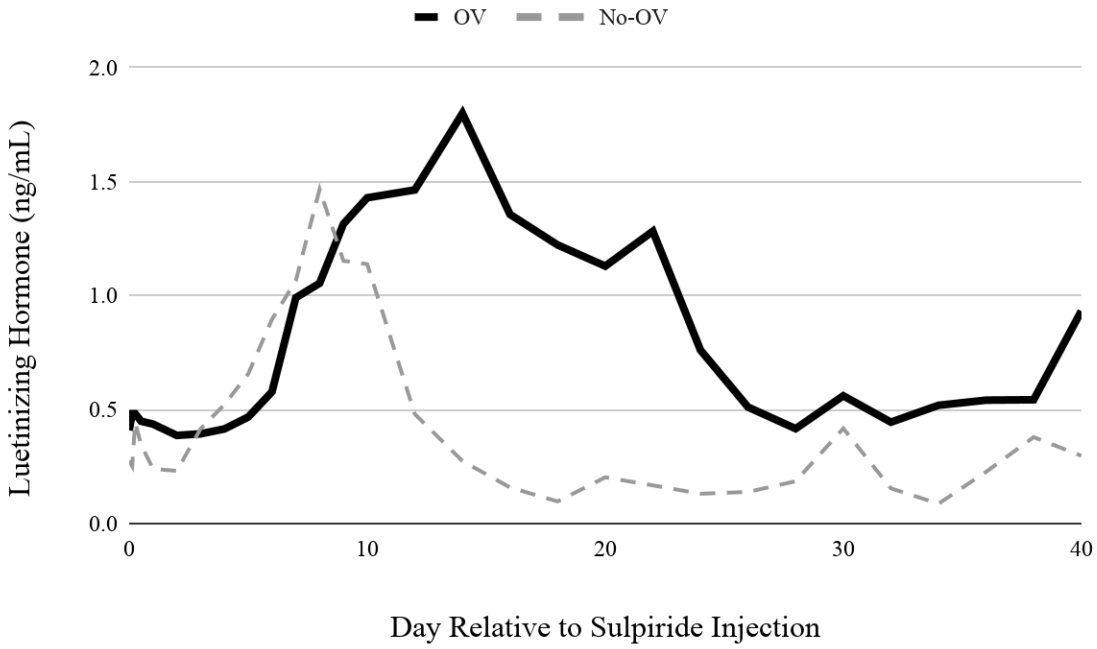

Figure 5. Mean plasma LH concentrations for treated mares that ovulated (OV) compared to mares that did not ovulate (No-OV) for 28 d after ECP and sulpiride injection.

OV, n = 23.
No-OV, n = 5.
Effect of the interaction of OV response x day, *P* = 0.248.
Effect of OV response, *P* = 0.130.
Effect of day, *P* = 0.007.

Treated mares: treated with ECP and sulpiride (Anestrous, Transitional, Progesterone groups)

Table A-1. Individual embryo transfer data and mare's pregnancy status at 60 d.

Mare	Treatment Group	Embryo Type	Cycle Number Transferred	Status at 60 d
3200	Progesterone	Frozen	2	Preg
9377	Transitional	Frozen	2	Preg
1342	Anestrus	Frozen	2	Open
2287	Transitional	Fresh	3	Preg
383	Transitional	Frozen	3	Open
7299	Anestrus	Fresh	4	Open
8351	Transitional	Clone	4	Preg
9357	Progesterone	Clone	5	Preg
9340	Transitional	Fresh	6	Preg
284	Progesterone	Fresh	5	Preg

Table A-2. Individual mare's number of days to first follicle greater than 20 mm, first and second ovulation, and days from first to second ovulation after ECP and sulpiride, or carrier injection.

Horse ID	Treatment Group	Number of days to follicle > 20 mm	Number of days to first ovulation	Number of days to second ovulation	Number of days from first to second ovulation
#1342	Anestrus	6	20	38	18
#278	Anestrus	8	10	-	-
#7299	Anestrus	6	15	36	21
#8342	Anestrus	8	-	-	-
#8355	Anestrus	6	15	-	-
#GSangria	Anestrus	13	-	-	-
Dolly	Anestrus	8	17	34	17
Sydney	Anestrus	6	20	43	20
Phoebe	Anestrus	8	10	-	-
Lynxy	Anestrus	6	13	41	28
Hummin	Anestrus	6	13	-	-
#383	Transitional	6	15	36	21
#8351	Transitional	3	8	36	28
V#275	Transitional	6	8	43	35
#2287	Transitional	6	8	17	9
#8320	Transitional	3	10	-	-
#9340	Transitional	3	6	20	14
#9377	Transitional	3	8	29	21
#GPinta	Transitional	8	10	41	31
#GV284	Progesterone	15	22	41	19
#GV283	Progesterone	8	-	-	-
#8346	Progesterone	6	20	-	-
#0554	Progesterone	6	13	-	-
#1510	Progesterone	6	-	-	-
#257	Progesterone	6	10	-	-
#9357	Progesterone	6	13	34	21
#2288	Progesterone	22	-	-	-
#3200	Progesterone	3	6	22	16
V Black	Control	10	48	-	-
#9134	Control	8	-	-	-
#2285	Control	8	45	-	-
#1321	Control	10	-	-	-

Table A-3. Individual mare's plasma LH concentrations by treatment for d 0 to 14.

Trt	Horse	0	0.125	0.25	0.5	1	2	3	4	5	6	7	8	9	10	12	14
T	383	0.395	0.459	0.444	0.053	0.140	0.120	0.249	0.207	0.033	0.122	3.425	0.722	1.722	1.885	3.014	5.044
T	2287	0.636	0.756	0.782	0.517	0.455	0.418	0.463	0.546	0.378	0.281	0.501	0.539	0.782	0.691	0.442	0.852
T	8320	0.065	0.057	0.115	0.010	0.033	0.058	0.350	0.283	0.070	0.440	0.089	0.087	0.140	0.077	0.160	0.038
T	8351	0.358	0.195	0.294	0.240	0.151	0.148	0.200	0.057	0.136	0.125	0.418	0.274	0.468	0.665	0.408	0.258
T	9340	0.563	0.463	0.312	0.359	0.275	0.131	0.236	0.411	0.401	0.345	0.838	0.701	0.534	1.094	2.153	1.005
T	9377	0.081	0.128	0.138	0.245	0.044	0.178	0.308	0.457	0.634	0.851	1.024	1.546	2.068	1.773	0.490	0.256
T	Pinta	0.358	0.821	0.411	0.263	0.256	0.471	0.274	0.858	0.240	0.190	0.150	0.368	1.127	1.483	2.859	4.095
T	V275	0.113	0.123	0.123	0.111	0.278	0.469	0.536	0.831	1.544	2.996	5.241	6.847	8.307	9.566	6.772	6.791
A	278	0.192	0.281	0.049	0.448	0.046	0.234	0.165	0.459	0.436	0.218	0.353	0.480	0.335	0.746	0.364	0.187
A	1342	0.198	0.217	0.244	0.439	0.274	0.180	0.232	0.259	0.288	0.274	0.440	0.679	0.383	0.587	0.535	0.544
A	7299	0.098	0.418	0.149	0.132	0.122	0.183	0.189	0.306	0.150	0.124	0.240	0.116	0.151	0.212	0.264	0.226
A	8342	0.432	0.398	0.440	0.314	0.365	0.298	0.274	0.469	0.238	0.312	0.319	0.377	0.316	0.372	0.304	0.202
A	8355	0.005	0.030	0.057	0.081	0.053	0.047	0.030	0.067	0.108	0.210	0.206	0.276	0.315	0.225	0.317	0.382
A	Dolly	0.111	0.211	0.052	0.098	0.063	0.052	0.355	0.195	0.280	0.582	1.211	2.121	4.167	2.787	5.356	10.63
A	Hum	0.139	0.067	0.065	0.306	0.116	0.187	0.245	0.517	0.985	1.944	3.231	4.071	3.991	4.350	4.159	2.316
A	Lynx	0.186	0.145	0.304	0.161	0.310	0.465	0.252	0.384	0.753	1.181	1.708	1.988	1.938	1.999	2.463	4.635
A	Phoe	0.160	0.209	0.295	0.340	0.261	0.230	0.541	0.316	0.567	0.329	0.339	0.427	0.481	0.317	0.484	0.209
A	Sang	0.421	0.394	0.489	0.334	0.255	0.130	0.312	0.203	0.262	0.108	0.698	0.289	0.321	0.424	0.565	0.375
A	Syd	0.152	0.194	0.290	0.162	0.073	0.118	0.363	0.145	0.363	0.205	0.150	0.270	0.216	0.039	0.108	0.209
P	257	0.223	0.098	0.063	0.106	0.262	0.234	0.213	0.082	0.108	0.108	0.053	0.085	0.069	0.099	0.105	0.108
P	554	0.016	-	-	-	0.111	0.068	0.097	0.342	0.368	0.429	0.709	0.814	0.839	0.828	1.010	0.367
P	1510	0.273	0.267	0.637	-	0.275	0.287	0.520	0.446	0.405	0.406	0.253	0.522	0.427	0.514	0.497	0.347
P	2288	0.220	0.144	0.635	0.519	0.231	0.401	0.911	1.415	2.197	3.486	3.942	6.043	4.631	4.302	0.913	0.404
P	3200	3.976	5.201	5.494	5.110	5.745	3.797	2.593	1.294	1.392	0.770	0.669	0.490	0.646	1.030	0.829	0.871
P	8346	0.180	0.134	0.130	0.182	0.182	0.312	0.202	0.315	0.344	0.413	0.413	0.381	0.295	0.438	0.237	0.168
P	9357	0.508	0.346	0.429	0.441	0.447	0.372	0.598	0.630	0.891	0.898	0.977	0.702	1.010	0.913	0.893	0.900
P	283	0.029	0.059	0.018	0.105	0.074	0.039	0.062	0.076	0.181	0.169	0.114	0.102	0.068	0.078	0.123	0.044
P	284	0.689	0.535	1.034	0.526	0.342	0.423	0.361	0.585	0.274	0.265	0.401	0.269	0.250	1.070	0.240	1.293
C	1321	0.124	0.116	0.064	0.170	0.272	0.207	0.030	0.283	0.168	0.903	0.072	0.426	0.241	0.246	0.123	0.275
C	2285	0.109	0.046	0.095	0.048	0.010	0.056	0.032	0.078	0.085	0.029	0.106	0.219	0.184	0.085	0.068	0.206
C	9134	0.289	0.349	0.231	0.277	0.581	0.563	0.304	0.263	0.367	0.346	0.109	0.143	0.236	0.113	0.047	0.228
C	black	0.099	0.275	0.161	0.115	0.159	0.222	0.117	0.141	0.125	0.167	0.095	0.098	0.117	0.180	0.234	0.162

Table A-4. Individual mare's plasma LH concentrations by treatment for d 16 to 40.

Trt	Horse	16	18	20	22	24	26	28	30	32	34	36	38	40
T	383	5.168	7.191	5.229	3.329	0.733	0.468	0.383	0.361	0.311	0.441	1.208	-	-
T	2287	1.344	2.731	1.563	1.068	0.929	0.541	-	-	-	-	-	-	-
T	8320	0.222	0.075	0.086	0.042	0.109	0.054	0.081	0.185	0.018	0.149	0.038	0.069	0.056
T	8351	0.491	0.338	0.254	0.093	0.138	0.162	0.252	0.294	0.409	0.768	1.596	-	-
T	9340	2.269	3.457	3.618	0.692	0.463	0.188	-	-	-	-	-	-	-
T	9377	0.305	0.174	0.126	0.166	0.239	0.251	0.261	-	-	-	-	-	-
T	Pinta	2.232	1.446	0.673	0.316	0.499	0.393	0.451	0.161	0.366	0.892	0.964	1.547	4.227
T	V275	1.752	0.568	0.355	0.314	0.265	0.165	0.291	3.016	1.900	1.928	0.467	0.417	0.412
A	278	0.215	0.069	0.289	0.167	0.095	0.043	0.413	0.183	0.177	0.481	0.158	0.287	0.189
A	1342	0.919	0.696	0.461	0.244	0.211	0.197	0.366	0.246	0.301	0.260	0.346	0.390	-
A	7299	0.129	0.135	0.163	0.164	0.118	0.023	0.018	0.088	0.112	0.032	0.039	0.055	-
A	8342	0.146	0.075	0.112	0.046	0.022	0.071	0.030	0.063	0.054	0.060	0.148	0.050	0.032
A	8355	0.215	0.194	0.182	0.140	0.247	0.197	0.176	0.184	0.153	0.181	0.274	0.184	0.143
A	Dolly	8.309	1.167	0.402	0.284	0.127	0.152	0.145	1.141	0.482	0.260	-	-	-
A	Hum	0.755	0.501	0.397	1.841	1.241	0.511	0.336	0.183	0.171	0.213	0.244	0.142	0.158
A	Lynx	1.335	0.619	0.457	0.344	0.291	0.144	0.225	1.263	1.019	0.709	0.584	0.584	1.082
A	Phoe	0.211	0.112	0.214	0.240	0.182	0.252	0.270	0.390	0.388	0.094	0.220	0.123	0.186
A	Sang	0.112	-	0.352	0.401	0.256	0.189	0.094	0.992	0.111	0.070	0.325	0.650	0.722
A	Syd	0.173	0.201	0.237	0.286	0.171	0.365	0.065	0.079	0.258	0.308	0.158	0.136	0.209
P	257	0.024	0.100	0.078	0.048	0.033	0.079	0.096	0.029	0.032	-.	0.087	-	0.019
P	554	0.100	0.204	0.206	0.215	0.677	0.395	0.573	0.204	0.081	0.049	0.217	0.199	0.224
P	1510	0.370	0.166	0.216	0.295	0.187	0.320	0.360	0.831	0.397	0.094	0.423	0.679	0.606
P	2288	0.132	0.095	0.209	0.092	0.172	0.060	0.376	0.109	0.129	0.102	0.198	0.382	0.011
P	3200	1.145	2.049	2.536	4.114	4.808	1.912	-	-	-	-	-	-	-
P	8346	0.131	0.123	0.146	0.105	0.151	0.221	0.172	0.204	0.215	0.084	0.189	0.151	0.189
P	9357	0.420	0.323	0.498	0.491	0.476	0.761	0.899	1.127	0.910	1.489	-	-	-
P	283	0.033	0.149	0.124	0.004	0.016	0.054	0.070	0.093	0.080	0.109	0.042	0.134	0.117
P	284	3.328	5.642	7.810	14.79	5.300	4.271	1.963	0.723	0.675	1.139	1.536	1.778	3.864
C	1321	0.173	0.087	0.175	0.216	0.376	0.063	0.182	0.157	0.153	0.283	0.217	0.135	0.219
C	2285	0.105	0.110	0.158	0.073	0.118	0.190	0.170	0.143	0.189	0.160	0.236	0.458	0.326
C	9134	0.269	0.184	0.126	0.188	0.126	0.129	0.193	0.106	0.051	0.022	0.204	0.101	0.094
C	black	0.136	0.118	0.305	0.661	0.142	0.122	0.116	0.428	0.471	0.081	0.232	0.126	0.128

Table A-5. Individual mare's plasma prolactin concentrations by treatment for d 0 to 4.

Trt	Horse	0	0.125	0.25	0.5	1	2	3	4
T	V275	0.212	28.637	11.453	9.488	6.829	11.486	6.992	7.295
T	383	1.358	11.47	5.476	3.784	6.479	3.537	3.187	4.432
T	2287	0.275	10.1	7.452	8.236	4.723	4.492	2.655	6.15
T	8320	0.972	19.232	9.253	2.143	2.656	2.982	1.094	1.478
T	8351	1.219	9.568	4.984	1.906	3.209	1.727	2.288	1.463
T	9340	7.07	32.454	11.47	2.286	1.464	3.51	2.42	2.697
T	9377	0.253	10.894	5.064	6.198	2.092	2.132	1.741	1.852
T	Pinta	1.439	26.008	11.561	3.387	4.297	5.662	3.717	2.692
A	1342	0.225	8.048	4.98	4.179	6.779	9.27	5.246	6.872
A	7299	-	16.055	8.02	3.549	2.732	2.109	1.277	1.188
A	8342	0.335	10.893	4.756	3.07	4.109	1.53	1.137	0.687
A	8355	1.38	8.492	17.144	3.351	4.408	4.797	2.694	2.145
A	Dolly	1.763	19.948	19.473	3.781	6.714	19.502	9.275	9.365
A	Hum	0.276	12.521	9.518	6.532	17.268	22.304	16.609	13.591
A	Lynx	0.833	14.559	7.54	6.631	6.056	5.152	4.733	1.563
A	Phoe	0.364	9.533	6.876	1.704	5.981	3.502	3.431	2.202
A	Sang	0.333	24.582	15.427	6.236	3.947	3.37	3.044	3.788
A	Syd	1.419	10.838	8.579	11.19	25.946	22.814	26.786	11.408
P	257	0.793	10.388	5.836	3.199	11.339	4.008	1.15	1.481
P	278	1.856	9.662	5.384	9.381	3.499	8.73	3.104	13.483
P	283	0.58	9.029	6.204	4.456	7.52	8.596	3.529	4.545
P	284	0.326	10.774	8.285	5.081	2.987	3.401	1.936	1.175
P	554	0.682	8.519	-	5.251	12.211	11.558	15.462	13.895
P	1510	3.289	10.177	8.877	.	14.22	12.467	12.823	8.369
P	2288	0.191	7.748	5.428	1.464	2.154	0.997	2.684	1.994
P	3200	1.093	21.184	14.494	12.916	9.67	6.864	3.929	2.75
P	8346	0.344	5.714	3.178	1.63	2.696	1.029	0.712	1.258
P	9357	0.597	17.975	10.57	20.933	17.451	59.833	12.039	6.568
C	1321	0.948	0.775	0.494	0.233	0.415	0.666	0.582	0.366
C	2285	0.706	1.363	0.977	0.41	0.18	0.118	0.121	0.145
C	9134	1.836	0.919	1.954	1.297	3.899	0.919	0.542	0.751
C	black	1.776	8.12	5.619	2.581	1.522	1.26	0.972	1.323

Table A-6. Individual mare's plasma prolactin concentrations by treatment for d 5 to 10.

Trt	Horse	5	6	7	8	9	10
T	V275	4.44	4.737	3.94	4.307	1.367	3.194
T	383	2.582	18.376	2.107	2.164	7.248	8.019
T	2287	14.505	3.153	2.267	2.181	3.167	5.737
T	8320	2.54	5.692	0.642	2.233	1.85	1.968
T	8351	2.659	7.614	0.837	0.56	0.872	1.98
T	9340	8.029	1.554	1.245	7.569	0.265	0.134
T	9377	3.573	2.21	0.291	0.473	0.013	0.434
T	Pinta	8.026	5.99	1.602	2.461	4.308	1.44
A	1342	4.655	5.802	2.685	4.85	3.22	2.219
A	7299	1.218	1.218	1.5	0.759	0.645	1.327
A	8342	2.185	3.002	1.478	1.947	1.559	1.363
A	8355	1.995	1.945	1.4	1.132	0.55	0.868
A	Dolly	12.6	14.016	10.84	11.289	9.985	6.533
A	Hum	10.914	9.079	9.256	7.419	5.172	3.743
A	Lynx	1.598	1.069	1.192	1.083	0.715	0.817
A	Phoe	0.996	4.272	2.181	1.759	0.88	0.873
A	Sang	1.885	3.238	1.533	1.573	2.378	1.541
A	Syd	6.511	6.583	4.118	2.993	2.274	1.998
P	257	3.24	6.73	2.799	1.396	4.748	3.401
P	278	9.15	7.046	3.816	4.871	6.555	4.99
P	283	3.897	5.63	1.202	0.801	0.206	0.201
P	284	0.592	1.378	1.008	0.732	0.747	2.711
P	554	10.969	7.276	6.089	5.695	3.497	0.167
P	1510	11.046	9.96	7.063	5.222	6.257	3.891
P	2288	1.124	2.592	0.688	1.267	1.448	1.185
P	3200	2.882	3.527	2.087	1.164	1.638	0.499
P	8346	1.049	2.444	0.913	0.879	1.532	1.447
P	9357	8.664	7.615	5.774	6.454	4.111	2.301
C	1321	1.042	1.537	0.257	0.011	0.142	0.103
C	2285	0.091	0.983	0.305	0.347	0.038	1.011
C	9134	3.931	2.83	0.699	0.366	1.197	1.523
C	black	2.576	2.017	0.343	1.314	1.745	1.361