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# Evaluation of a Role for Prolactin in the Recrudescence of Ovarian Activity in Seasonally Anovulatory Mares

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EVALUATION OF A ROLE FOR PROLACTIN IN THE RECRUDESCENCE OF OVARIAN  
ACTIVITY IN SEASONALLY ANOVULATORY MARES

A Dissertation

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
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in

The School of Animal Sciences

by

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This dissertation is dedicated to the memory of my grandfather, Donald G. Henson, who passed away on March 12, 2016. In my eyes, my grandfather was the epitome of all things wonderful. His kindness, generosity and extraordinary work ethic have always inspired and will continue to inspire me to lead a similar life. From him, I inherited the recognition that honor, generosity and integrity, not wealth, power or egotism, are one's legacy. I wish I could share this achievement with him on Earth, but I know that his pride in me and love for me will be celebrated in Heaven.

“There is joy in work.” – Don Henson

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## **ABSTRACT**

A series of experiments studied the possible role for prolactin in the spring-time recrudescence of ovarian activity in mares. The first experiment was based on cabergoline administration to seasonally anovulatory mares to prevent the seasonal rise in endogenous plasma prolactin to assess whether reduced prolactin altered onset of ovarian activity or first ovulation. Although prolactin concentrations were reduced, basal prolactin concentrations recovered from suppression by the time of the next injection (10 days later), even though low-dose sulpiride stimulation of prolactin was still suppressed by 85%. The subsequent experiment tested whether season affected the duration and degree of suppression produced by a standard dose of cabergoline. Mares were administered cabergoline at 1.5 mg/500 kg body weight in March, June, September, and December. Duration of suppression was affected little by month of assessment. Follow-up assessments with either low dose sulpiride (July) or thyrotropin releasing hormone (October) challenges after administration of 5 mg cabergoline indicated that basal prolactin concentrations always rebounded earlier than secretagogue-induced secretion. The third experiment studied possible physical and hormonal characteristics of seasonally anovulatory mares that might affect their response to a combined estradiol and sulpiride treatment for inducing ovulation in winter. All mares received 50 mg of estradiol cypionate (ECP) followed by 3 g of sulpiride. Factors that were commonly associated with early-induced ovulation were: adequate body condition, elevated plasma concentrations of leptin and insulin, and a greater LH response to secretagogue prior to the start of the experiment. The final experiment studied the hormonal production aspects of the first pre-ovulatory follicle of the breeding season in seasonally anovulatory mares treated with ECP followed by injections of sulpiride 5 and 12 days later, or ECP plus vehicle (controls), beginning in January. Date to first 35-mm follicle was

advanced in sulpiride-treated mares. It was concluded that early-induced follicles are equally as competent in terms of steroid production and ability to respond to circulating LH as pre-ovulatory follicles occurring naturally in spring. These experiments support the model in which an increase in circulating prolactin during winter can hasten follicular growth and advance the date to first ovulation in most mares.



## **CHAPTER 1: INTRODUCTION**

An industry-imposed birthday of January 1 for most registered horse breeds has created an incentive for horse owners and breeders to produce foals as early in the year as possible. However, this becomes difficult due to the mare's long gestation period and the seasonal nature of the mare's reproductive cycle. Mares in Louisiana typically enter a period of reproductive inactivity, known as seasonal anestrus, in late October and do not return to cyclicity until early April. Therefore, advancing the first ovulation of the season is desirable in order for breeders to produce maximally competitive foals, i.e., born close to the January 1<sup>st</sup> birthdate, and has led to years of study of spring transition in mares and numerous attempts to develop pharmacological therapies for inducing early cyclicity.

Much effort is invested yearly in the effort to manipulate the mare's reproductive cycle so she ovulates in mid-February. The common method currently used in the field to induce early cyclicity is artificial lighting. This has proven to be labor intensive and cost prohibitive for most farms. Of the pharmacological therapies used tested to date, the most promising is the use of prolactin (a pituitary hormone) or dopamine antagonists such as sulpiride or domperidone that stimulate prolactin. Elevated prolactin during seasonal anestrus stimulates follicular growth and hastens ovulation in most, but not all, mares. It is unclear how prolactin induces early ovulation, but it is suspected that prolactin acts directly on the ovary.

Ten years of experimentation at Louisiana State University has revealed success rates (percentage of mares ovulating early) ranging from 50 – 90% in mares treated with a combination of estrogen and either domperidone, or sulpiride. The failure of all mares (100%) to respond to treatment led to the current research projects, which were designed to identify an obligatory role for prolactin during vernal transition and assess factors that might affect whether or not a mare responds to stimulated prolactin with early ovulation.

An understanding of the ovarian changes that occur in the presence of prolactin will allow researchers to identify cause-and-effect relationships between prolactin and ovarian function. This knowledge could help generate new or perfect protocols to induce early cyclicity in more mares. This could allow for enhanced breeding practices such as initiating cyclicity in embryo donor and recipient mares, obtaining oocytes earlier for artificial reproductive technologies or for natural breeding programs that wish to cycle mares earlier in the year.

## **CHAPTER 2: REVIEW OF LITERATURE**

### **2.1 Overview**

General aspects of mare reproductive physiology have been extensively reviewed in my master's thesis [2.1], and are not repeated here for the sake of brevity. Details regarding ovarian anatomy, cyclicity, seasonality of reproduction, and the role of prolactin in reproduction are also covered in that document, as well as in the review article by Thompson and Oberhaus [2.2]. The aim of this literature review is to address the most recent information regarding the use of estrogen plus dopaminergic antagonists to hasten the date to first ovulation in seasonally anovulatory mares, to detail the role of dopamine in reproduction in mares, and to evaluate a role for prolactin in ovarian function in the horse.

A detailed account of the effects of prolactin and dopamine antagonists on ovarian activity in seasonally anovulatory mares, and on hair growth and shedding can be found in the review by Thompson and Oberhaus [2.2]. To summarize, several decades worth of research has focused on using dopamine antagonists, particularly sulpiride, to stimulate prolactin in an effort to hasten the vernal transition in mares. Given that the seasonal pattern of prolactin secretion (low during winter vs. high during summer [2.3-2.5]) parallels seasonal reproduction in mares, researchers hypothesized that stimulating prolactin at a time when it is naturally low would return mares to reproductive cyclicity.

### **2.2 Use of Estradiol and Anti-dopaminergic Compounds**

Anti-dopaminergic agents such as sulpiride and domperidone release the adenohipophysis from hypothalamic dopaminergic suppression by blocking dopamine receptors on pituitary lactotropes, thus stimulating prolactin release [2.6]. Kelley et al. [2.7] demonstrated a greater prolactin response to sulpiride and thyrotropin releasing hormone (TRH) in mares pre-

treated with estradiol benzoate compared to mares receiving sulpiride alone. Treatment with estradiol benzoate also increased plasma luteinizing hormone (LH) concentrations, which, coupled with increased prolactin secretion, hastened the date to first ovulation in treated mares [2.7]. Estradiol is a known stimulator of prolactin synthesis in pituitary lactotropes in many species [2.8-2.10]. Thompson et al. [2.11] demonstrated a marked increase in pituitary content of prolactin in pony mares treated with estradiol, suggesting a direct influence of estradiol on prolactin synthesis.

Thompson et al. [2.12] determined that, in geldings, one injection of estradiol cypionate (ECP) provided prolactin responses similar to those after several injections of estradiol benzoate (same as those used by Kelley et al. [2.7]). Given the similar response, one injection of ECP was chosen for further experimentation. Mitcham et al. [2.13, 2.14] compared prolactin and ovarian responses in mares receiving ECP (50 or 100 mg) combined with domperidone (1.5 g) either 1, 6, or 11 days after ECP treatment. Success, as defined by ovulation within 28 days of treatment, was similar in mares receiving either 50 or 100 mg of ECP, but was greater in mares receiving domperidone 1 day later vs. 6 days or 11 days later. No effect of ECP dosage on circulating prolactin was observed; however, prolactin in mares receiving domperidone 1 day later tended to be higher when compared to days 6 and 11, although prolactin was stimulated at these times as well. Mitcham et al. [2.13] also compared domperidone (1.5 g in biodegradable microparticles, i.m.) with varying doses (0.75 and 1.5 g, i.m.) of a new, non-particle formulation of sulpiride in ECP treated mares. Treatment with domperidone took place 1 day after treatment with 100 mg ECP; treatment with sulpiride took place 1, 6 and 11 days post treatment with 100 mg ECP. The prolactin response was similar in mares treated with either 0.75 g or 1.5 g sulpiride formula. Mares that received domperidone or 0.75 g sulpiride did not ovulate earlier compared to

controls; however, of the mares treated with the higher dose of sulpiride (1.5 g), 7 of the 9 ovulated earlier relative to controls. Although domperidone did stimulate prolactin, the response tended to be lower than mares treated with sulpiride. Given the better prolactin response, as well as its commercial availability, sulpiride has been chosen as the dopamine antagonist of choice for subsequent studies conducted at Louisiana State University. Previous results have shown that the effect of sulpiride on prolactin secretion is quick but not as long lasting as domperidone. Therefore, either repeated injections of sulpiride are needed or development of a long acting vehicle is necessary.

The ovarian results, albeit variable, have been mostly favorable. Kelley et al. [2.7] reported a success rate (percentage of mares ovulating within a specified period of time) of 89%; Mitcham [2.15] reported on multiple experiments, with the lowest success rate being 50%. In all studies, mares not responding initially to the treatment regimen basically had ovulation dates similar to mares not treated with a dopamine antagonist. It is this failure of a given mare to respond with ovarian activity that has raised the question as to what factors may be involved in her lack of response, or conversely, what factors contribute to the positive responses in mares that do in fact respond to treatment with estrogen and a dopaminergic antagonist.

### **2.3 Role of Dopamine in Reproduction**

The hypothalamic-pituitary axis is regarded as the control center regulating all reproductive events, including puberty, estrous cyclicity, ovulation, and seasonal reproduction. This involves input from both intrinsic and extrinsic factors to the hypothalamus which, in turn, releases or inhibits release of gonadotropin releasing hormone (GnRH) accordingly. However, a substantial amount of research has shown that the mammalian ovary receives direct neural inputs via sympathetic nerves in addition to its hormonal inputs via the circulatory system [2.16-2.19].

For example, denervation of the rat ovary results in delayed puberty, minimal follicular growth and reduced estradiol secretion [2.20-2.21]. In the mare, dopaminergic and adrenergic nerves have been observed in the uterus and cervix [2.22]. Moreover, Welsh [2.23] detected catecholaminergic neurons in whole ovaries from anestrus and summer cycling mares. Antibodies to tyrosine hydroxylase (TH) or dopamine- $\beta$ -hydroxylase (D $\beta$ H), enzymes involved in conversion of tyrosine to dopamine and dopamine to norepinephrine, respectively, were used to immunolocalize neurons. Positive immunoreactive signals were present for TH and/or D $\beta$ H in all ovaries. The majority of neurons were positive for TH but not D $\beta$ H, suggesting those neurons were dopaminergic. In addition, King et al. [2.24] immunolocalized dopamine receptors in both equine granulosa/theca cells as well as in the corpus luteum. Furthermore, ovarian cortex as well as luteal tissue expressed D1 and D2 receptor messenger RNA (mRNA); a lower level of expression was detected in the theca and granulosa layers of the follicle [2.24, 2.25]. Local release and potential action of dopamine is evident at the level of the ovary; however, its mechanism of action in cyclicity has yet to be determined in the mare.

Involvement of catecholamines, particularly dopamine, in reproduction has been investigated in a number of species. Dopamine and dopamine-like compounds appear to have anti-gonadal effects in teleost fish [2.26, 2.27], sheep [2.28, 2.29], and cows [2.30, 2.31]. Dopamine agonists have also been studied as a treatment to prevent progression of ovarian hyperstimulation syndrome in women receiving fertility treatments [2.32, 2.33] as well as to treat infertility associated with hyperprolactinemia [2.34].

Dopamine-like compounds, such as the ergot alkaloids found in endophyte infected fescue grass, have well-documented deleterious effects on lactation and parturition in mares. Mares grazing endophyte infected fescue during the last 60 days of gestation experience

agalactia, prolonged gestation, hypertrophic and/or retained placental tissues, increased occurrence of dystocia and still birth [2.35, 2.36]. The role of dopamine and dopamine-like compounds in other aspects of mare reproduction such as estrous cyclicity have not been clearly described.

Bennett-Wimbush et al. [2.37] treated three groups of seasonally anestrous pony mares with perphenazine (dopamine antagonist) twice daily, bromocriptine (dopamine agonist) daily, or vehicle from January 20 until ovulation. Perphenazine advanced the first ovulation by 30 days compared to controls, while bromocriptine had no effect on time to first ovulation but did appear to delay growth of preovulatory sized follicles. Similarly, Bass [2.38] administered a long acting dopamine agonist, cabergoline, orally during the natural breeding season, which suppressed prolactin concentrations but had little to no effect on ovulation or luteal progesterone production. Interestingly, both Bennett-Wimbush et al. [2.37] and Bass [2.38] reported erratic and prolonged estrous behavior in mares treated with dopamine agonists both during the non-breeding and breeding seasons. While circulating prolactin concentrations were suppressed in both studies, neither daily bromocriptine nor oral cabergoline were able to completely suppress prolactin for any length of time.

## **2.4 Regulation of Prolactin by Dopamine**

Unlike most other adenohypophyseal hormones, which rely on hypothalamic releasing factors for secretion, prolactin secretion by lactotropes in the adenohypophysis is regulated through tonic inhibition by hypothalamic dopamine [2.39]. Axons from dopaminergic neurons terminate in the median eminence of the adenohypophysis, release dopamine into the hypophysial portal system where it reaches the adenohypophysis by way of long portal vessels and binds to dopamine receptors on the plasma membrane of lactotropes [2.40]. Five distinct

dopamine receptors (D1 through D5) have been identified and can be grouped into two subgroups: D1-like, which includes D1 and D5 receptors, and D2-like, which includes D2, D3 and D4 receptors [2.40].

Receptor variants D1 and D2 are expressed at higher levels and are more selective for agonists and antagonists. It is through D2 receptors that dopamine primarily regulates prolactin secretion [2.41]. Activation of D2 receptors on lactotropes results in both immediate changes in membrane permeability, which prevents release of prolactin from secretory granules, as well as gradual changes in cell machinery, such as inhibition of adenyl cyclase, decrease in inositol phosphates and eventual decrease in prolactin gene expression [2.40]. Given the heterogeneous nature of lactotropes [2.42-2.45], it is unclear whether these events occur in all cell types in the same manner and at the same rate.

## **2.5 Subpopulations of Lactotropes**

The ultrastructure of pituitary lactotropes has been described in several species [2.42-2.49]. Nogami et al. [2.46] reported four morphologically different lactotrope populations in the male rat pituitary which differed based on the size of the cell as well as the size of secretory granules. In most species studied, the majority of prolactin secreting cells were described as large and containing large, dense secretory granules [2.47-2.49]; however, others have also reported the findings of smaller prolactin-secreting cells which contained smaller granules [2.50, 2.51]. Few studies have determined differential secretory activity of these different cell types.

Christian et al. [2.51] described three morphological subtypes (Type I, II and III) of prolactin-secreting cells in the rat pituitary. By examining exocytosis of prolactin granules, it appeared all three types were inhibited by dopamine, but only Type II and Type III lactotropes



were stimulated with TRH or vasoactive intestinal peptide (VIP), another known prolactin secretagogue.

Rahmanian et al. [2.45] described morphological differences in two types of lactotropes within the equine pituitary, Type I and II. Type I lactotropes were generally larger with large, dense secretory granules, much like those observed in other species, and were indistinguishable from the cells that contained both growth hormone and prolactin, the mammosomatotropes. Type II lactotropes were generally smaller than Type I with smaller secretory granules. Both cell types stained positive for prolactin; however, it is not known if both cell types secrete prolactin similarly in response to secretagogues or inhibitors [2.45].

## **2.6 Cabergoline**

In humans, the dopaminergic agonist pergolide was previously chosen for its long duration of prolactin suppression (48 hr after a single 50 µg dose) [2.52] until the advent of cabergoline. Cabergoline has been shown to be an even more potent and long acting dopaminergic agonist with a single dose of 200 µg lasting 96 hr and 600 µg lasting 168 hr [2.53]. Recently, Hebert et al. [2.54] reported complete suppression of plasma prolactin concentrations in mares and geldings with 5 mg of cabergoline in a slow releasing vehicle injected intramuscularly. Even when challenged with a low dose of sulpiride 10 days after cabergoline injection, prolactin remained suppressed. Magnitude and duration of suppression were considerably greater in cabergoline treated horses compared to pergolide treated horses [2.54]. Additionally, Arana Valencia et al. [2.55] administered a total of 7 cabergoline injections 10 days apart and demonstrated no incidences of refractoriness to cabergoline in mares challenged with sulpiride (one day before the next cabergoline injection) or side effects to the cabergoline compound.

## **2.7 Role of Prolactin in Regulation of Ovarian Gene Expression**

Aside from its well-documented effects on lactation, prolactin has gained considerable recognition as a hormone that supports other reproductive processes such as ovulation, formation and maintenance of the corpus luteum (CL), and implantation. Prolactin has also been demonstrated to be a permissive hormone which facilitates apoptosis and regression of the CL in cycling rats [2.56, 2.57].

In regards to changes in ovarian gene expression in response to prolactin, most studies have been conducted using the rat as a model. In rats, prolactin appears to be obligatory for proper CL formation and maintenance [2.58, 2.59]. More specifically, it has been suggested that prolactin is responsible for inducing functional LH receptors on granulosa cells and CL of rats as well as sustaining progesterone secretion from the CL. In rat granulosa cells, prolactin has been shown to sustain concentrations of LH receptors, but not without first being induced by FSH [2.60]. Similar to these findings are the reports of Richards and Williams [2.61] and Holt et al. [2.62] who observed an enhancive effect of prolactin on LH receptor content and progesterone production, but not a direct effect of prolactin alone. Conversely, Bjurulf et al. [2.63] observed a ten-fold increase in LH receptor messenger mRNA in luteal cells of prolactin-treated rats as well as an increase in circulating progesterone concentrations when compared to controls. This effect of prolactin on LH receptors and receptor mRNA in females is directly analogous to the complete requirement for prolactin for spermatogenesis in the male hamster [2.64, 2.65], which was shown to be mediated by prolactin's necessity for LH receptors on hamster Leydig cells [2.66].

One of the greatest arguments for a role for prolactin in ovarian function is the aberrations observed in null mutant prolactin receptor mice: they display multiple reproductive

defects including infertility, low ovulation rates, decreased fertilization rates and a markedly reduced number of primary follicles [2.67]. Binart et al. [2.68] also observed reproductive deficiencies in prolactin receptor knockout mice such as failure of the CL to produce progesterone and failure of embryo implantation which was rescued by progesterone administration. Grosdemouge et al. [2.69] failed to observe any differences in length of estrous cycle, ovulation rates or ovarian response to gonadotropins in null mutant prolactin receptor mice; however, they did observe irregularities in corpus luteum morphology such as highly disorganized cells, early regression and extremely low vascularization, which they determined led to luteal insufficiency. Furthermore, a marked decrease in LH receptor mRNA was detected in null mutant mice. These mice also displayed a decrease in mRNA for P450scc, a steroidogenic enzyme responsible for converting cholesterol to pregnenolone in the first steps of steroid synthesis [2.69].

In the mare, a local role for prolactin in the ovary has been proposed due to the remarkable and rapid growth of ovarian follicles in response to either exogenous prolactin or dopamine antagonists during seasonal anestrus. Exactly how exogenous or endogenously stimulated prolactin facilitates early follicular growth has yet to be determined. King et al. [2.70] and Oberhaus et al. [2.71] located receptors for prolactin on equine luteal cells and ovarian granulosa and theca cells, suggesting that prolactin can bind to these cell types and elicit a response.

## **2.8 Rationale for Present Experiments**

Researchers continue to strive to understand the mechanism by which the mare is able to transition in and out of cyclicity in order to provide breeders with a convenient and fail-safe technique for inducing earlier cyclicity. An increase in circulating prolactin has obvious

stimulatory effects on the mare ovary during seasonal-induced acyclicity. More detailed studies investigating the relationship between prolactin and ovarian stimulation could provide a clearer understanding of the mechanism by which prolactin is involved in seasonal transition.

The goals of the experiments presented herein were 1) to assess any perturbations in vernal transition due to complete suppression of prolactin with cabergoline, 2) to determine any seasonal variation in ability of cabergoline to suppress prolactin, 3) retrospectively, to determine factors that might affect whether or not a mare responds to a combined estradiol-sulpiride treatment, and 4) to assess factors, both molecular and hormonal, that change in response to a combined estradiol-sulpiride treatment. It was hypothesized that 1) suppression of prolactin would delay vernal transition, 2) prolactin response to cabergoline would vary across seasons, 3) factors such as body condition and nutritional status would modulate an ovarian response to estradiol and sulpiride, and 4) treatment with estradiol and sulpiride, and hence prolactin, would upregulate LH receptor mRNA in ovarian cells in seasonally anovulatory mares.

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## **CHAPTER 3: EFFECT OF REPEATED CABERGOLINE TREATMENT ON THE VERNAL TRANSITION AND HAIR SHEDDING OF MARES (YEAR 1) AND A SUBSEQUENT COMPARISON OF THE EFFECT OF STARTING DATE ON PROLACTIN SUPPRESSION (YEAR 2)<sup>1</sup>**

### **3.1 Summary**

Two studies were conducted to determine efficacy of cabergoline for suppressing prolactin (PRL) and the possible effects on vernal transition in mares. In Experiment 1, six mares each received either vehicle or cabergoline (5 mg, intramuscularly) every 10 days for 12 treatments beginning February 4, 2013. Blood samples were drawn regularly and mares were challenged with sulpiride periodically to assess PRL suppression. Weekly hair samples were obtained to determine shedding. Prolactin was suppressed ( $P < .05$ ) by cabergoline, but suppression waned in spring. There was no effect ( $P > .1$ ) of treatment on day of first ovulation, LH or FSH. Hair shedding was generally suppressed ( $P = .05$ ). In 2014 (Experiment 2), 8 of the same 12 mares were used in a similar experiment to determine if the rise in PRL observed in Experiment 1 was due to refractoriness to cabergoline or perhaps another factor. Treatment began on April 6, 2014, corresponding to the increase in PRL in treated mares in Experiment 1. Mares were treated with cabergoline or vehicle until June 5. Prolactin was suppressed ( $P < .05$ ) by cabergoline and the pattern of apparent escape from suppression was similar to year 1. We conclude that 1) cabergoline at this dose alters hair shedding but does not alter the time of first ovulation in mares, and 2) relative to our previous reports of cabergoline treatment in the fall, there is a seasonal effect on the ability of this dose of cabergoline to suppress unstimulated prolactin secretion.

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## 3.2 Introduction

Prolactin appears to be involved in the vernal transition of seasonally anestrous mares, given that administration of exogenous prolactin [3.1, 3.2] or a dopamine antagonist [3.3-3.5] can initiate early follicular growth and hasten the date to first ovulation. Besognet et al [3.3] treated mares with the dopamine antagonist, sulpiride, and were able to increase prolactin concentrations and advance the first ovulation of the season by approximately 20 days when compared to untreated mares. Subsequent researchers have tested both sulpiride and domperidone with varying degrees of success, advancing the first ovulation of the season by as much as 40 days [3.5]. In addition to the effects on reproduction, elevated prolactin appears to also have a stimulatory effect on hair coat shedding [3.3, 3.6].

The mechanisms involved with the ovarian response to prolactin in seasonally anovulatory mares have not been elucidated. Localization of receptors for prolactin on equine granulosa and theca cells [3.7] as well as the presence of prolactin in follicular and luteal tissue [3.8, 3.9] are supportive of a model in which prolactin acts directly on the equine ovary during the vernal transition. Although much research has been devoted to administering dopamine antagonists, few experiments have described the effects of prolactin suppression on the vernal transition in mares. Bennett-Wimbush [3.10] treated three groups of anestrous pony mares with perphenazine twice daily, bromocriptine daily, or vehicle from January 20 until ovulation. Perphenazine, a dopamine antagonist, advanced the first ovulation by 30 days compared to controls, while the dopamine agonist, bromocriptine, had no effect on time to first ovulation, but did appear to delay growth of preovulatory sized follicles. Similarly, Bass [3.11] administered a long acting dopamine agonist, cabergoline, orally during the natural breeding season, which suppressed prolactin concentrations but had little to no effect on ovulation or luteal progesterone

production. While both studies suppressed circulating prolactin concentrations, neither daily bromocriptine nor oral cabergoline were able to completely suppress prolactin for any length of time.

Recently, Hebert et al. [3.12] reported complete suppression of prolactin using cabergoline in a slow releasing vehicle injected intramuscularly. Even when challenged with a low dose of sulpiride 10 days after cabergoline injection, prolactin remained suppressed. Additionally, Arana Valencia et al. [3.13] administered a total of seven cabergoline injections 10 days apart and demonstrated no incidences of refractoriness to cabergoline in horses challenged with sulpiride (one day before the next cabergoline injection) or side effects to the cabergoline compound. Based on those results, we hypothesized (Experiment 1) that cabergoline in the same vehicle used in the experiments conducted by Hebert et al. [3.12] and Arana Valencia et al. [3.13] would suppress prolactin in the long term and thus allow a better assessment of the need of circulating prolactin in follicular growth and eventual ovulation in mares transitioning from winter anovulation to a breeding season state in the spring. Given the less-than-total suppression of prolactin by cabergoline in late spring in Experiment 1, Experiment 2 was performed to test whether the lack of suppression was due to 1) a refractoriness of the mares to long-term cabergoline exposure, or perhaps 2) some seasonal change in the mares made them less susceptible to cabergoline suppression.

### **3.3 Materials and Methods**

Procedures used in these experiments were approved by the Institutional Animal Care and Use Committee of the Louisiana State University Agricultural Center.

### **3.3.1 Animals and treatments**

All mares in the two experiments were routinely maintained outdoors on native grass pasture during the warmer months, and were grazed on winter ryegrass pasture when available in late winter. In the period between availability of summer grasses and winter ryegrass, hay prepared from the same native grasses was provided for *ad libitum* consumption as needed.

Prior to the start of the first experiment, all non-pregnant mares in the resident herd were assessed by ultrasonic scanning of the ovaries once a week for three weeks starting January 20, 2013, and samples of jugular blood were collected every 4 days. Anovulation was defined as the absence of any follicle >20 mm, the absence of any corpora lutea, and plasma progesterone concentrations consistently less than 1 ng/mL.

### **3.3.2 Experiment 1**

Twelve light horse, anovulatory mares were identified and were allotted into two similar groups based on age (4 to 23 years old), body condition score (4 to 7) and ovarian follicular growth prior to start of the experiment (February 4, 2013). The groups were then randomly assigned as treatment (n = 6) and control (n = 6).

On February 4, 2013, and every 10 days thereafter for a total of 12 injections, mares assigned to the treatment group received 5 mg of cabergoline (Attix Pharmaceuticals, Toronto, Ontario, Canada) intramuscularly in a slow release vehicle (1 mL). Mares assigned to the control group received vehicle only (1 mL), intramuscularly, on the same schedule. The vehicle was a proprietary mixture of hydrophobic, oily liquids designed to provide sustained, slow release of cabergoline over time (Provided by Richard M. Gilley, BioRelease Technologies LLC, Birmingham, AL).

### **3.3.3 Experiment 2**

Eight of the same 12 mares from Experiment 1 were available and were used the following year. Mares remained in the same treatment groups in both experiments. On April 6, 2014, and every 10 days thereafter, mares in the treatment group (n = 4) received cabergoline as described in Experiment 1. Mares assigned to the control group (n = 4) received vehicle only. A total of seven treatment injections were given, with the last injection on June 5, 2014.

### **3.3.4 Ultrasonography and Estrous Behavior**

In Experiment 1, ovarian activity was monitored via ultrasonography (Aloka 550V with 5-Mhz linear-array transducer; Hitachi-Aloka, Wallingford, CT) once a week until a follicle >25 mm emerged. Once a follicle exceeded 25 mm, the mare was scanned daily until the follicle ovulated or regressed to <25 mm.

Also upon detection of a follicle >25 mm, the mare was checked for displays of estrus with one of two stallions daily until 2 days after her second ovulation. A single evaluator graded receptivity of the mare using a -3 to 3 scale, whereby, -3 = extreme aggression toward stallion, -2 = ear pinning, -1 = avoidance of stallion, 0 = indifferent to stallion, 1 = raising tail, 2 = raising tail as well as clitoral eversion, 3 = posturing and urinating.

In Experiment 2, only the prolactin response to cabergoline was characterized. Given the lack of effect on reproduction in Experiment 1, ultrasonography, estrus detection and circulating gonadotropins were not assessed in Experiment 2.

### **3.3.5 Blood Sampling**

**3.3.5.1 Experiment 1.** Jugular blood samples were collected in 10-mL evacuated tubes containing sodium heparin (Vacutainer, Becton and Dickinson, Franklin Lakes, NJ) beginning on Feb 5, 2013 (day 1), and every 4 days after until May 28 (day 113) to determine circulating

prolactin concentrations. Additionally, on the day of first ovulation and for 16 successive days after, a single blood sample was drawn to determine circulating concentrations of FSH, LH, and progesterone. Plasma was harvested by centrifugation at 1200 x g for 15 minutes and was stored at -20°C.

On days 29, 59, 79, 89 and 119 of the experiment, which was always nine days after an injection of cabergoline, mares were administered a low dose of sulpiride (2 µg/kg of body weight of a racemic mixture; Sigma Chemical Co., St. Louis, MO) intravenously in saline. Jugular blood was collected at 0, 5, 10 and 20 min relative to treatment to determine the prolactin response to sulpiride. Blood collection and storage was done in the same manner described above.

**3.3.5.2 Experiment 2.** Jugular blood samples were collected as described in Experiment 1 beginning on Apr 7, 2014 (day 1), and every other day after until June 14 (day 69), to determine circulating prolactin concentrations. Similar to Experiment 1, mares were challenged with a low dose of intravenous sulpiride on days 29 (May 5) and 69 (June 14) and blood collected at 0, 5, 10 and 20 min relative to treatment. Plasma was harvested and stored as described for Experiment 1.

### **3.3.6 Hair Collection**

In Experiment 1, once weekly for eight weeks beginning February 6, a tuft of hair was pulled three times from the left shoulder of each horse, stored, and later weighed to determine any differences in shedding of the winter coat. Hair was easily pulled from horses that were beginning to shed their winter coat and remained intact in horses with retained winter coats. On day 92 (May 7), a 3 x 3-cm patch of hair from approximately 6 inches below where the back meets the loin (rib area) was shaved with clippers and weighed. Due to a later start date in

Experiment 2, mares were already beginning to shed their winter coat, thus hair shedding was not assessed.

### **3.3.7 Sample and Data Analyses**

Due to the different objectives of the two experiments, data collection in Experiment 1 was more extensive than for Experiment 2. Specifically, Experiment 2 only monitored plasma prolactin concentrations in routine blood samples and after sulpiride challenge. All data collection and analyses are described herein.

Frozen plasma samples were thawed and analyzed for prolactin, FSH, LH, and progesterone as appropriate. Prolactin, FSH, and LH were measured by radioimmunoassay in assays previously validated by our laboratory [3.14-3.16]. Intra- and interassay coefficients of variation and levels of detection were 7%, 12% and 0.2 ng/mL for prolactin; 6%, 9%, and 0.2 ng/mL for LH; and 7%, 11%, and 1.4 ng/mL for FSH. Progesterone was analyzed using commercially available kit reagents (ImmuChem Double Antibody, <sup>125</sup>I RIA Kit, MP Biomedicals, Inc, Costa Mesa, CA).

Data for dependent variables collected over many different time points (plasma concentrations of prolactin, LH, FSH, and progesterone, and hair weight) were analyzed by one-way analysis of variance (ANOVA) with repeated sampling using the general linear model of SAS (SAS Instit., Cary, NC). Data for prolactin concentrations obtained during sulpiride challenges were analyzed as a double-split-plot design, with treatment as the main effect, repetitive challenges as the first repetition, and multiple sampling times within each challenge as the second repetition. Day of first ovulation was analyzed using a one-way ANOVA. When appropriate, differences between treatment groups within time periods were tested for significance by the least significant difference test [3.17].



Experiment 1 versus 2 similarities and differences in prolactin concentrations were analyzed by one-way ANOVA (year as the main effect) with repetitive sampling. Separate analyses were run for just control mares and just treated mares in both years. Differences between years within time periods were assessed by the least significant difference test [3.17].

### **3.3 Results**

#### **3.3.1 Experiment 1**

Towards the end of the first experiment, one mare in the control group developed severe lameness unrelated to the experiment and was subsequently euthanized. Her data for LH, FSH, progesterone, day of ovulation, and the first three sulpiride challenges were complete. There was no data for her in the final (fourth) sulpiride challenge, as well as the last 9 days of daily samples; she was included in the analyses otherwise.

There was a significant interaction between treatment and day ( $P < .0001$ ) for mean plasma prolactin concentrations in samples obtained every 4 days (Figure 3.1). Prolactin concentrations were generally low in both groups and not different ( $P > .1$ ) through day 61 (April 6, 2013). Thereafter, as prolactin concentrations gradually increased in control mares through day 113, those in cabergoline-treated mares decreased and then gradually rose again with each cabergoline injection, such that they were suppressed ( $P < .05$ ) relative to controls for the first one or two blood samples after injection, but not the entire 10-day period.

Mean day of first ovulation did not differ ( $P > .1$ ) between control mares (April  $7 \pm 7$  days) and treatment mares (April  $18 \pm 7$  days). Mean plasma LH, FSH, and progesterone concentrations in samples obtained daily from the day of first ovulation until day 16 are

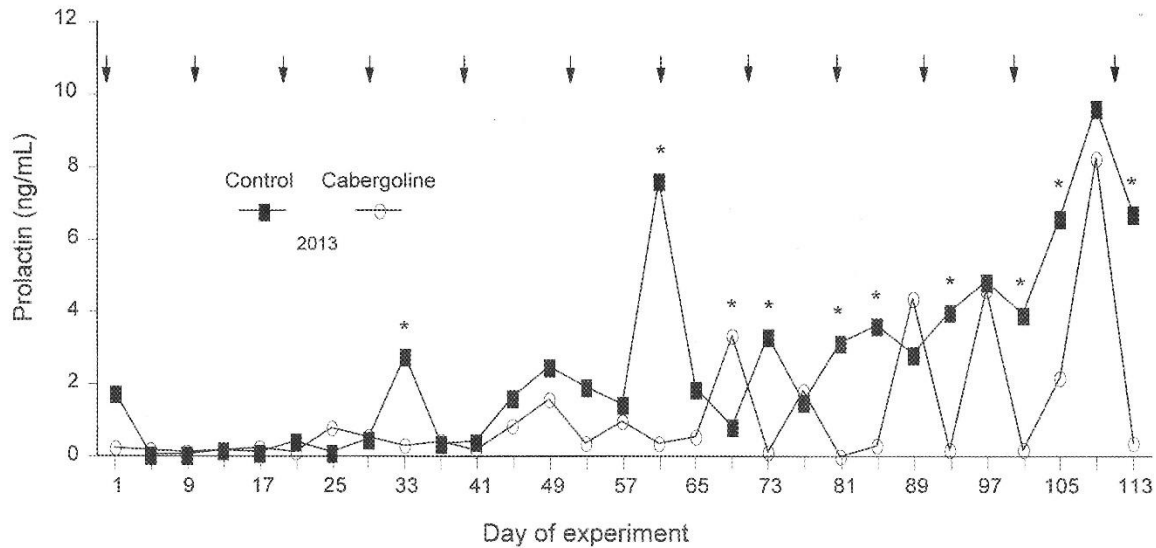


Fig. 3.1. Mean plasma concentrations of prolactin in Experiment 1 in samples obtained every 4 days from mares treated with 5 mg of the dopamine agonist, cabergoline (n = 6), or vehicle (controls; n = 6) every 10 days (treatment days indicated by arrow) from February 4, 2013 (day 0), through May 28, 2013 (day 113). After April 6 (day 61), prolactin in mares treated with cabergoline continued to decline with treatment; however, the effects did not last 10 days. A significant treatment by day interaction ( $P < .0001$ ) was detected and differences are indicated with asterisks. Pooled standard error of the mean from the analysis of variance was 1.0 ng/mL.

presented in Figure 3.2. Luteinizing hormone and FSH concentrations did not differ ( $P > .1$ ) between cabergoline and vehicle treated mares, nor were any differences found in circulating progesterone concentrations. Mean interovulatory intervals were not different between treated ( $24 \pm 0.67$  days) and control ( $24.5 \pm 1.9$  days) mares; no perturbations in estrous behavior were observed.

A robust prolactin response to sulpiride was observed in mares receiving vehicle only (Figure 3.3) and was greatly suppressed ( $P < .01$ ) in cabergoline-treated mares. With each subsequent sulpiride challenge, the prolactin response gradually increased in cabergoline-treated mares, although it remained greatly suppressed ( $P < .01$ ) when compared to control mares.

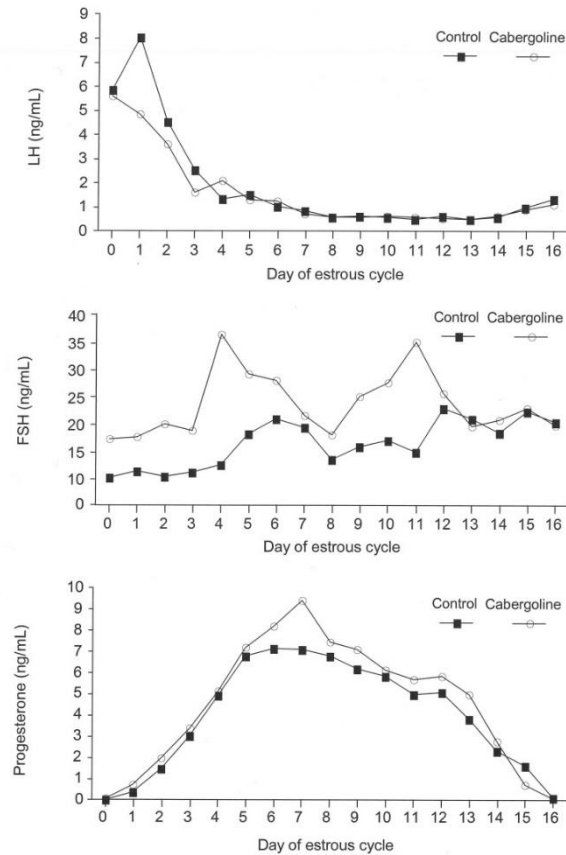


Fig. 3.2. Mean plasma concentrations of LH, FSH, and progesterone in samples collected in Experiment 1 on the day of first ovulation (day 0 for each mare) and for 16 successive days thereafter in mares treated with 5 mg of cabergoline ( $n = 6$ ) and mares treated with vehicle (controls;  $n = 6$ ). No differences ( $P > .1$ ) were detected between groups for any hormone. Pooled standard errors of the mean from the analyses of variance were 0.73, 6.8, and 1.1ng/mL for LH, FSH, and progesterone concentrations, respectively.

Weight of hair samples obtained weekly generally weighed less ( $P = .05$ , Figure 3.4) in cabergoline-treated mares than in control mares; however, weight of hair sample shaved from the rib area on May 7 did not differ between treated and control mares.

### 3.3.2 Experiment 2

Mean prolactin concentrations in samples obtained every other day from April 6 until June 13 are presented in Figure 3.5. Prolactin was suppressed ( $P < .05$ ) in mares treated with

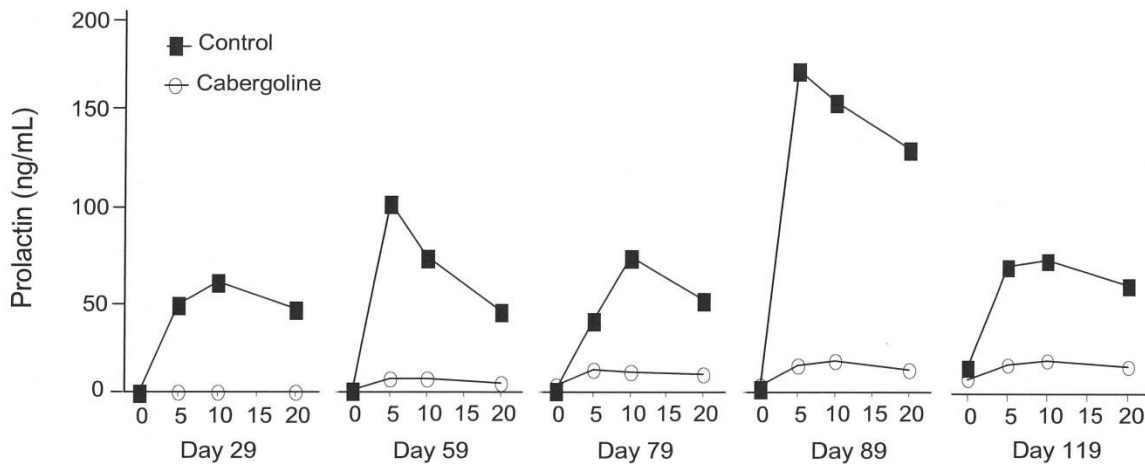


Fig. 3.3. Mean prolactin response to sulpiride in cabergoline- and vehicle-treated (control) mares on days 29, 59, 79, 89 and 119 in Experiment 1. Numbers within numbered days on the horizontal axis represent minutes relative to sulpiride treatment. Prolactin response to sulpiride was greatly suppressed ( $P < .01$ ) in cabergoline-treated mares. At each challenge, differences ( $P < .01$ ) between groups occurred at all time points except for the pre-treatment (0 min) sample. Pooled standard error of the mean from the analysis of variance was 3.3 ng/mL.

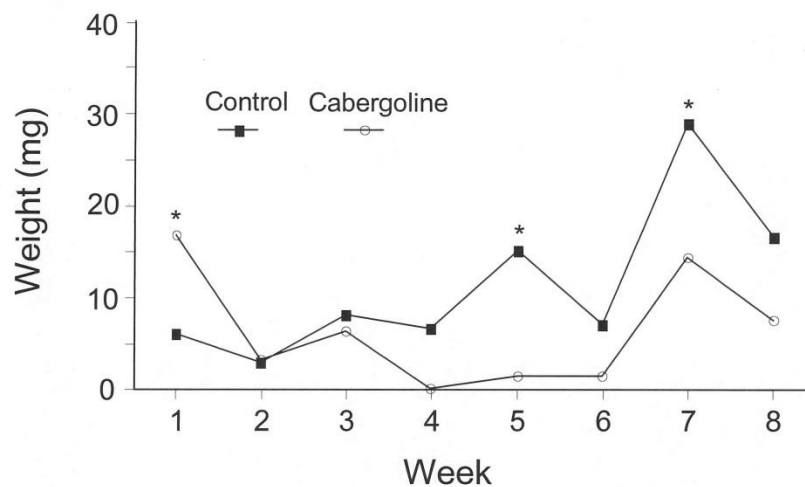


Fig. 3.4. Weight of hair samples obtained weekly in Experiment 1 for 8 weeks beginning February 6, 2013. Hair samples generally weighed less ( $P = .05$ ) in cabergoline-treated mares than in controls. Differences are indicated by the asterisks. Pooled standard error of the mean from the analysis of variance was 5.7 mg.

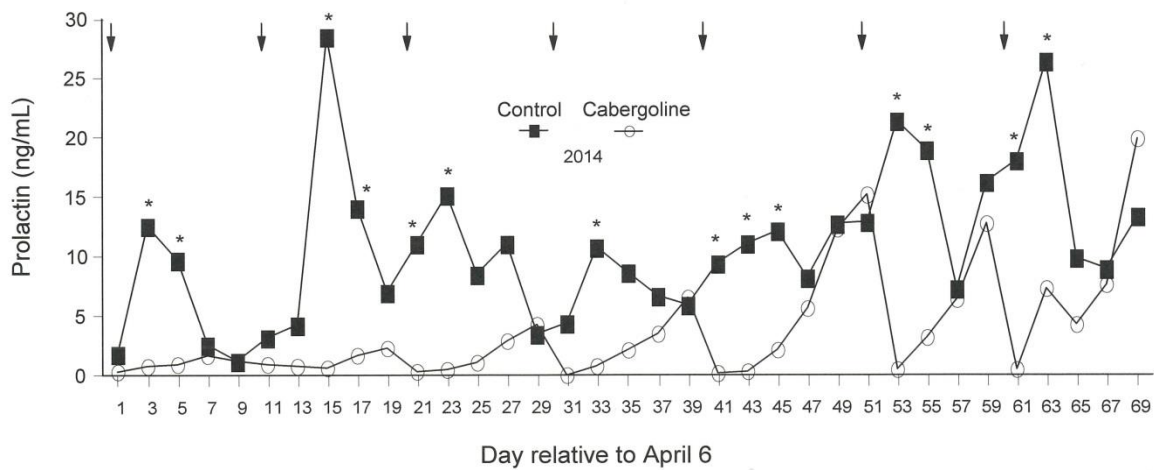


Fig. 3.5. Mean plasma concentrations of prolactin in Experiment 2 in samples obtained every other day from mares treated with 5 mg of cabergoline ( $n = 4$ ) or vehicle (controls;  $n = 4$ ) every 10 days (treatment days indicated by arrow) from April 6, 2014 (day 0) through June 5, 2014 (day 69). Prolactin concentrations were mostly suppressed ( $P < .05$ ) in cabergoline treated mares except in samples near the next cabergoline injections. Differences between groups within days are indicated by the asterisks. Pooled standard error of the mean from the analysis of variance was 4.5 ng/mL.

cabergoline, with the pattern of suppression being very similar to Experiment 1. Prolactin response to sulpiride on day 29 (May 4) and day 69 (June 14) was greatly suppressed ( $P < .01$ ) in mares treated with cabergoline, while vehicle-treated mares had a robust response to sulpiride. When super-imposing the sulpiride challenges in 2014 over the same sulpiride challenge days in 2013 (Figure 3.6), the prolactin response to sulpiride is very similar, particularly in cabergoline-treated mares.

Analysis of the two separate years in a single ANOVA revealed that prolactin in vehicle-treated mares varied ( $P < .05$ , Figure 3.7) from Experiment 1 (2013) to Experiment 2 (2014), with unstimulated prolactin being generally higher in 2014. The pattern of prolactin suppression in cabergoline-treated mares, however, was very similar ( $P > .1$ , Figure 3.8) from year to year.

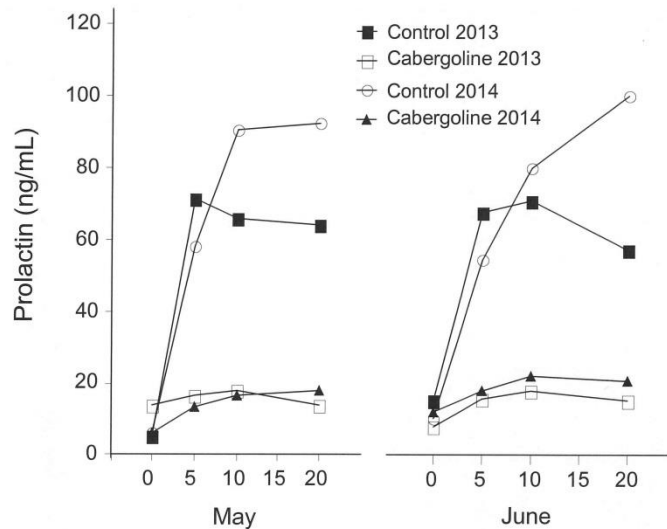


Fig. 3.6. Comparison of prolactin response to sulpiride in May and June, 2013 and 2014, in cabergoline and vehicle-treated mares. Numbers on the horizontal axis represent minutes relative to sulpiride treatment. Prolactin responses to sulpiride were suppressed ( $P < .01$ ) in mares treated with cabergoline. Differences between cabergoline and vehicle-treated mares occurred at all time points except pre-treatment (0 min) samples. The suppressive effect of cabergoline on prolactin concentrations after sulpiride was similar for the two years. Pooled standard error of the mean from the analysis of variance was 10.3 ng/mL.

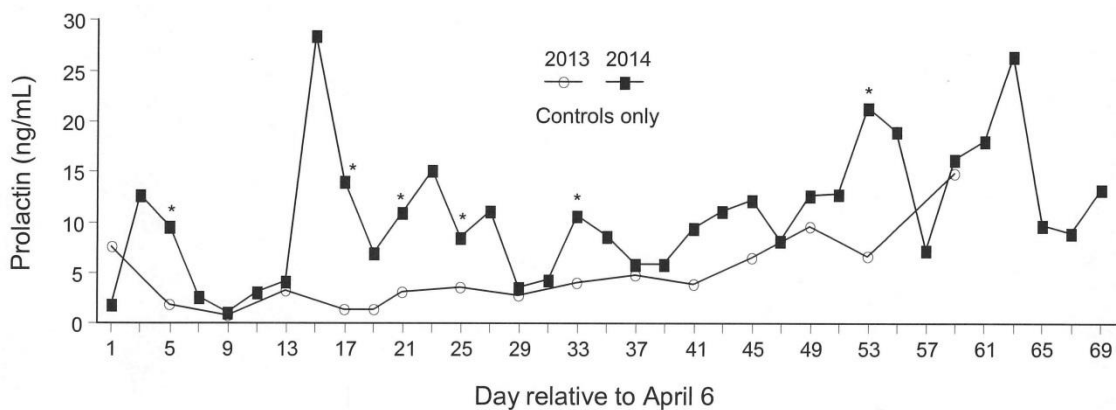


Fig. 3.7. Comparison of the unstimulated plasma prolactin concentrations in control mares only over the two years of experimentation (2013 versus 2014). Samples were aligned relative to April 6 for both years. Plasma prolactin in control mares varied ( $P < .05$ ) from year to year, with those in Experiment 2 being generally higher than in Experiment 1. Differences are noted by the asterisks. In Experiment 2, plasma samples were collected more frequently and for a longer period of time than in Experiment 1, which accounts for the greater number of data points in 2014. While all data are presented in the graph, only data points from 2014 that had a matching data point on the same day from 2013 were included in the analysis of variance. Pooled standard error of the mean from the analysis of variance was 2.3 ng/mL.

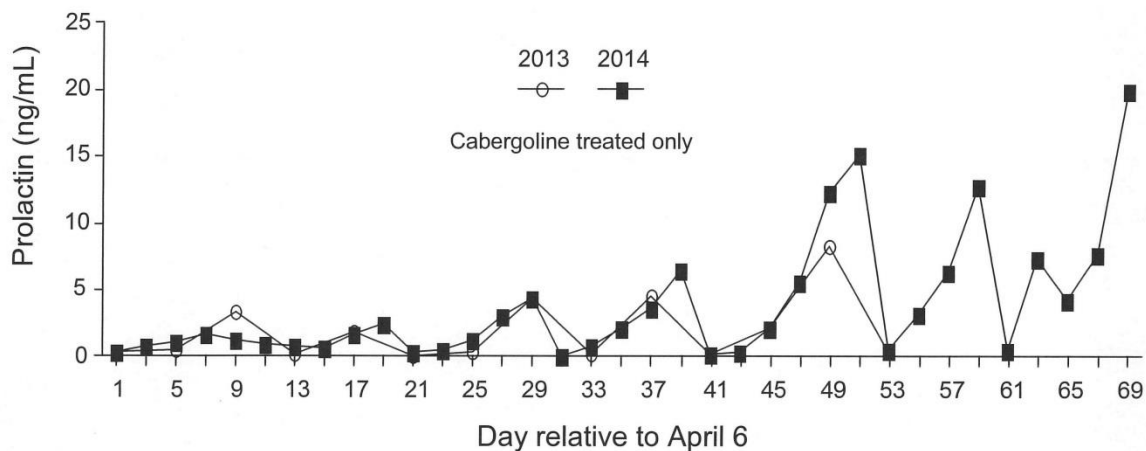


Fig. 3.8. Comparison of the unstimulated plasma prolactin concentrations in cabergoline-treated mares only over the two years of experimentation (2013 vs 2014). Samples were aligned relative to April 6 for both years. Plasma prolactin in mares treated with cabergoline were similar between year 1 and 2. In Experiment 2, plasma samples were collected more frequently and for a longer period of time than in Experiment 1, which accounts for the greater number of data points in 2014. While all data points are presented in the graph, only data points from 2014 that had a matching data point on the same day from 2013 were included in the analysis of variance. Pooled standard error of the mean from the analysis of variance was 1.7 ng/mL.

### 3.4 Discussion

Given the ability of exogenous prolactin [3.1, 3.2], as well as endogenous prolactin stimulated by dopaminergic antagonists [3.3-3.6], to hasten ovulation in seasonally anovulatory mares, the question arises as to what role prolactin actually plays in the normal vernal transition period in mares. If prolactin was an obligatory factor for ovulation, then removing it from the circulation would be expected to either prevent or at least delay the date of first ovulation. Our hypothesis, based on results of experiments done in the fall of the year [3.12, 3.13], was that 5 mg of cabergoline in the slow-release vehicle used in those experiments would totally suppress prolactin secretion during treatment. Prolactin concentrations were generally suppressed in Experiment 1 up to April 6, the time of normally expected first ovulation at the LSU AgCenter

Horse Farm (personal observation). Thus, although our original hypothesis concerning cabergoline's suppression of prolactin was not supported (for later time periods after April), the suppression of prolactin prior to April 6 had no effect on date of first ovulation. Prolactin concentrations in vehicle-treated mares began to rise around March 17 (day 41) with a large peak of prolactin occurring on April 6 (day 61). Mean first day of ovulation for vehicle-treated mares was April 7. Similarly, prolactin in cabergoline-treated mares increased on April 14 (day 69) and mean first day of ovulation for treated mares was April 18. Given that mean first day of ovulation for both groups of mares occurred just after a rise in prolactin, it could be that mares recovered from cabergoline suppression quickly enough such that sufficient prolactin was available for normal follicular development and ovulation. Alternatively, it could be that circulating prolactin is not an obligatory factor for follicular growth and ovulation in these mares at this time. Most of the data collection for LH and FSH during the post-ovulatory cycle was in April, when prolactin concentrations were no longer totally suppressed, thus the lack of treatment effect could be due to adequate prolactin being available, or to a lack of involvement of prolactin in the secretion of these gonadotropins. The same can be said of progesterone concentrations in the first diestrous period.

A problem encountered in human medicine of using dopaminergic agonists for appetite suppression was gradual resistance to the agonist, or tolerance to its effects [3.18, 3.19], which required subsequently higher dosages to achieve the same suppression. If refractoriness to cabergoline was the cause of the eventual rise in prolactin in April and thereafter in Experiment 1, we would have expected a period of total suppression in treated mares for approximately 60 days after start of treatment (well into June) during the second year (Experiment 2) followed by a rise in prolactin as observed the previous year. Although there was considerable variation in



prolactin concentrations from year 1 to year 2, that variation can be attributed to the control mares (Figure 3.7). The patterns of suppression in cabergoline-treated mares in years 1 and 2 were almost identical (Figure 3.8), indicating that a refractoriness to cabergoline treatment was not likely the cause of an eventual rise in prolactin. Therefore, other physiological changes, such as seasonal change(s) in other factor(s), need to be considered.

Clavier et al. [3.20] reported a seasonal variation in dopaminergic input to the adenohypophysis in mares based on the mean sulpiride dose required to counteract the endogenous dopamine input and to achieve a 50% maximal prolactin response during spring, summer, fall and winter. Mean 50% maximal dose was lowest in June, which would equate to a lower endogenous dopaminergic input during that season relative to the others. Mean 50% maximal doses were highest in September and December. Although any reduced input of endogenous dopamine to the lactotropes in the spring should have been overwhelmed by the exogenous cabergoline in Experiment 1, the original dose of 5 mg every 10 days, which was totally suppressive of prolactin in the fall [3.12, 3.13], may have become inadequate after April 6. In addition to seasonal variations in dopamine input to the adenohypophysis, seasonal changes in lactotrope sensitivity to dopamine (perhaps reduced receptors) is a possibility [3.21]. Interestingly, the prolactin response to sulpiride after April 6 was still greatly suppressed in cabergoline-treated mares even when unstimulated (every four-day samples) prolactin had begun to rise. This may indicate a long-term, and continuing, suppression of releasable stores of prolactin separate from the stores available for daily (basal) release. Alternatively, if indeed dopamine receptors are reduced at this time due to some seasonal factor, then the response to blockage of those receptors (by sulpiride) may be reduced (less sensitive) as well. Assessment of

total prolactin content in the adenohypophysis and measurement of dopamine receptor numbers during this period are needed to help explain our current observations.

Hair coat shedding was perturbed by cabergoline treatment in Experiment 1. This is consistent with other observations [3.3, 3.6] in which pronounced shedding of winter coat was observed in mares treated with sulpiride (resulting in increased prolactin secretion) or with recombinant porcine prolactin [3.2]. Weight of hair pulled once weekly tended to weigh less in cabergoline-treated mares, suggesting retention of winter coat. Unfortunately, no pre-treatment hair samples were collected for weight determination; however, visual inspection clearly revealed a retained winter coat in treated mares well into late spring and even early summer. Similarly, Thompson et al [3.2] reported retention of patches of winter hair coat as late as May in pony mares that had developed high antibody titers against exogenous recombinant porcine prolactin. The fact that winter hair shedding was slowed in mares in Experiment 1 after April is an indication that prolactin suppression was indeed causing a biological response. Apparently, hair shedding is more sensitive to perturbations in plasma prolactin concentrations than is follicular growth, ovulation, and the associated hormonal secretion rates.

In conclusion, cabergoline as administered in the present experiments was effective in suppressing prolactin secretion, however, complete or near complete suppression was only achieved in very early spring; thereafter, inhibition of prolactin still occurred, but the duration of suppression waned at the dose of cabergoline and the vehicle used. Refractoriness does not appear to be the cause of the prolactin escape from cabergoline. It is likely that an unknown physiological factor, such as seasonal variation in dopaminergic input [3.20], or perhaps sensitivity to dopamine [3.21], is over-riding the inhibitory dopaminergic effect of cabergoline,

given that the pattern of prolactin escape was almost identical from year 1 to year 2. No negative effect on reproduction was observed, which is consistent with other reports [3.10, 3.11].

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## **CHAPTER 4: SEASONAL ASSESSMENT OF CABERGOLINE SUPPRESSION OF PROLACTIN IN MARES: UNSTIMULATED VERSUS SULPIRIDE STIMULATED AND THYROTROPIN RELEASING HORMONE STIMULATED RESPONSES**

### **4.1 Summary**

Six experiments were performed to assess a possible seasonal variation in prolactin suppression from administration of cabergoline. Experiments 1-4 were carried out near the vernal equinox, summer solstice, autumnal equinox and winter solstice, respectively. For each of those experiments, 10 light horse mares ( $n = 6$  cabergoline;  $n = 4$  vehicle) were administered 1.5 mg/500 kg BW cabergoline in oil or oil only, i.m. Blood samples were drawn at -10 minutes, 0, 1, 3, 6, 12 hours, and every 12 hours thereafter until 168 hours (7 days). Cabergoline suppressed ( $P < .001$ ) prolactin in Experiments 1 and 2 with prolactin returning to concentrations similar to controls around 120 hours (5 days). In Experiment 3 (October), prolactin was suppressed ( $P < .001$ ) in cabergoline-treated horses compared to vehicle-treated mares at 3, 6, 36, 108, 156 and 168 hours post treatment. Experiment 4 (December) revealed differences ( $P < .001$ ) between cabergoline- and vehicle-treated mares only at 6 and 120 hours post treatment, given that prolactin concentrations are naturally low in winter. In Experiment 5 (July), 13 mares were treated ( $n = 5$  vehicle;  $n = 4$  compounded cabergoline;  $n = 4$  cabergoline in oil) and blood was drawn as described for Experiments 1-5 but continued until 264 hours; sulpiride challenges (.01 mg/kg BW, i.v.) were administered every-other-day. Cabergoline suppressed ( $P < .0001$ ) prolactin in both cabergoline formulation groups and the patterns of suppression were virtually identical. Unstimulated prolactin returned to concentrations similar to controls around 132 hours; however, the prolactin response to low dose sulpiride continued to remain greatly suppressed ( $P < .0001$ ) even after unstimulated levels had returned to normal. In Experiment 6 (October), 6 mares each received either 5 mg cabergoline in oil or oil only, and blood was drawn regularly

until 264 hours; TRH challenges (2 mg/500 kg BW, i.v.) were administered every-other-day. Cabergoline suppressed ( $P < .05$ ) prolactin in treated mares for at least 96 hours compared to controls; however, at that time, circulating levels of prolactin in controls dropped remarkably and were indistinguishable from cabergoline-treated mares. The prolactin response to TRH was completely suppressed ( $P < .05$ ) in treated mares through 120 hours, but began to recover in subsequent challenges. The recovery of TRH-stimulated prolactin was observed earlier than sulpiride-stimulated prolactin in Experiment 6. In conclusion, no seasonal variation was observed for the duration of suppression or the degree of suppression of prolactin by a fixed dose of cabergoline. However, both sulpiride and TRH-stimulated prolactin continued to be suppressed in cabergoline-treated mares long after unstimulated prolactin concentrations had returned to normal. This apparent dichotomy is consistent with a model of at least two subpopulations of lactotropes regulated differentially by dopaminergic input, as has been reported in other species.

## **4.2 Introduction**

Cabergoline is a potent dopamine receptor agonist that acts upon dopaminergic type 2 (D2) receptors [4.1]. In humans, cabergoline is used frequently in low doses to treat hyperprolactinemia [4.2, 4.3] and does not appear to be associated with deleterious side effects, such as heart valve dysfunction reported for other dopamine receptor agonists such as pergolide and bromocriptine [4.4-4.7].

Hebert et al. [4.8] and Arana Valencia et al. [4.9] assessed the effects of cabergoline on plasma prolactin in mares and geldings as a potential replacement therapy for pergolide in the treatment of pituitary pars intermedia dysfunction (PPID) in horses. Both authors began cabergoline treatments during the fall and reported complete suppression of prolactin in the face

of low dose challenges with the dopamine antagonist sulpiride. A single injection of 5 mg of cabergoline suppressed sulpiride-induced prolactin for 10 days [4.8]. In comparison, 2 mg of pergolide, the currently available drug for PPID treatment, reduced prolactin for 24 hours when injected and only 12 hours when given orally [4.8]. Subsequently, Arana Valencia et al. [4.9] administered a total of 7 cabergoline injections 10 days apart beginning in October and demonstrated no incidences of refractoriness to cabergoline in horses challenged with sulpiride (one day before the next cabergoline injection) or side effects to the cabergoline compound.

Given the remarkable magnitude and duration of prolactin suppression by cabergoline, Oberhaus et al. [4.10] administered 10 treatments 10 days apart to seasonally anovulatory mares starting in February to assess possible perturbations in vernal transition. Blood sampling every 4 days initially revealed low concentrations of prolactin in both treated and control groups, as would be expected in mares during late winter. In early spring, as plasma prolactin concentrations rose to detectable levels, cabergoline suppressed prolactin in treated mares; however, the suppression gradually waned before the next treatment. In a repeat experiment the following year, Oberhaus et al. [4.10] determined that the lack of 10-day suppression was not due to refractoriness to cabergoline, and suggested that other cues, such as season, may influence the prolactin response to dopaminergic agonists. In the same two experiments, even after the suppression of unstimulated prolactin had waned, the prolactin response to low dose sulpiride injection was still 85% suppressed when compared to control mares [4.10].

Given these conflicting patterns of prolactin suppression, the series of trials described herein was designed 1) to assess any potential seasonal variation in the prolactin response to cabergoline, 2) to compare suppressive effects of cabergoline in oil to cabergoline in a known, slow-releasing vehicle and 3) to evaluate differences in prolactin stimulation in cabergoline-

treated horses to sulpiride and TRH, two known prolactin secretagogues that act via distinct, separate receptor systems.

### **4.3 Materials and Methods**

Procedures used in these experiments were approved by the Institutional Animal Care and Use Committee of the Louisiana State University Agricultural Center.

#### **4.3.1 Animals**

All mares used in these experiments were maintained outdoors on native grass pasture during the warmer months, and were grazed on winter ryegrass pasture when available in late winter. In the period between availability of summer grasses and winter ryegrass, hay prepared from the same native grasses was provided for *ad libitum* consumption as needed.

#### **4.3.2 Experiments 1-4**

Ten light horse mares ranging in age from 6 to 25 years old and weighing 380 to 550 kg were treated with an intramuscular injection of either cabergoline (n = 6; 1.5 mg/500 kg BW) or vehicle only (controls; n = 4). Cabergoline (Sigma-Aldrich, St. Louis, MO) was dissolved in a minimal amount of diethyl ether and then mixed into vegetable oil (Crisco; J.M. Smucker Company, Orrville, OH). Vehicle-treated mares received oil only with ether added.

Experiments 1-4 began on March 22, July 15, October 8 and December 27, 2015, respectively. Ten minutes prior to treatment, a single blood sample was collected from each mare via jugular venipuncture into 6-mL evacuated tubes containing sodium heparin as an anticoagulant (Vacurette, Greiner Bio-One, Monroe, NC). Blood sampling was continued at 0, 1, 3, 6, 12 hours and then every 12 hours after cabergoline and vehicle injections until 168 hours (7 days). For all blood samples collected throughout the experiment, plasma was harvested by centrifugation at 1200 x g for 15 minutes and was subsequently stored at -20°C.



#### **4.3.3 Experiment 5**

On July 16, 2016, 13 light horse mares of similar ages and weights as described above received a pretreatment intravenous injection of sulpiride in saline (0.01 mg/kg BW; Sigma-Aldrich, St. Louis, MO) and blood was drawn at 0, 10 and 20 minutes post treatment. The following day, mares were assigned to treatment groups and received either 5 mg of cabergoline in oil as described above (n = 4), 5 mg of cabergoline (Attix Pharmaceuticals, Toronto, Ontario, Canada) in a proprietary slow release vehicle (n = 4), or oil only (n = 5). All injections were 1 mL. The slow release vehicle was a proprietary mixture of hydrophobic, oily liquids designed to provide sustained, slow release of cabergoline over time (Provided by Richard M. Gilley, BioRelease Technologies LLC, Birmingham, AL). Blood samples were drawn at -10 minutes, 0, 1, 3, 6, and 12 hours and then every 12 hours after until 264 hours (11 days). One day after treatment as well as every other day until day 11, all mares were challenged with intravenous low dose sulpiride as described above. Plasma was harvested by centrifugation at 1200 x g for 15 minutes and was stored at -20°C.

#### **4.3.4 Experiment 6**

On October 16, 2016, 12 light horse mares of similar ages and weights as described above received a pretreatment intravenous injection of thyrotropin releasing hormone (TRH, 2 mg/500 kg BW; Sigma-Aldrich, St. Louis, MO) in saline. Blood was collected 0, 10, 20 and 30 minutes relative to injection. The following day, mares assigned to treatment group (n = 6) received 5 mg cabergoline in oil, intramuscularly, while control mares (n = 6) received oil only. Preparation of cabergoline in oil was the same as described in the first 5 experiments. Blood was sampled at 0, 1, 6, and 24 hours and then every 24 hours thereafter until 264 hours (day 11). One day after treatment as well as every other day until day 11, all mares were challenged with

intravenous TRH as described above. Plasma was harvested by centrifugation at 1200 x g for 15 minutes and was stored at -20°C.

#### **4.3.5 Sample and Data Analysis**

Frozen plasma samples were thawed and analyzed for prolactin by radioimmunoassay previously validated for equine samples by Colborn et al. [4.11]. Intra- and interassay coefficients of variation and minimal levels of detection for prolactin were 7%, 12% and 0.2 ng/mL.

Data for plasma concentrations of prolactin, collected over sequential time points, were analyzed by one-way analysis of variance (ANOVA) with repeated sampling using the general linear model of SAS (SAS Instit., Cary, NC). Data for prolactin concentrations obtained during sulpiride and TRH challenges were analyzed in double-split-plot design ANOVA, with treatment as the main effect, repetitive challenges as the first repetition, and multiple sampling times within each challenge as the second repetition. When appropriate, differences between treatment groups within time periods were tested for significance by the least significant difference test [4.12].

#### **4.4 Results**

Mean plasma concentrations of prolactin for Experiments 1-4 are presented in Figure 4.1. Cabergoline effectively suppressed ( $P < .001$ ) prolactin in treated horses in Experiment 1 and 2 with prolactin returning to concentrations similar to controls around 120 hours. In Experiment 3 (October), prolactin was suppressed ( $P < .001$ ) in cabergoline-treated horses compared to vehicle-treated mares at 3, 6, 36, 108, 156 and 168 hours post treatment. Experiment 4 (December) revealed differences ( $P < .001$ ) between cabergoline- and vehicle-treated mares only at 6 and 120 hours post treatment.

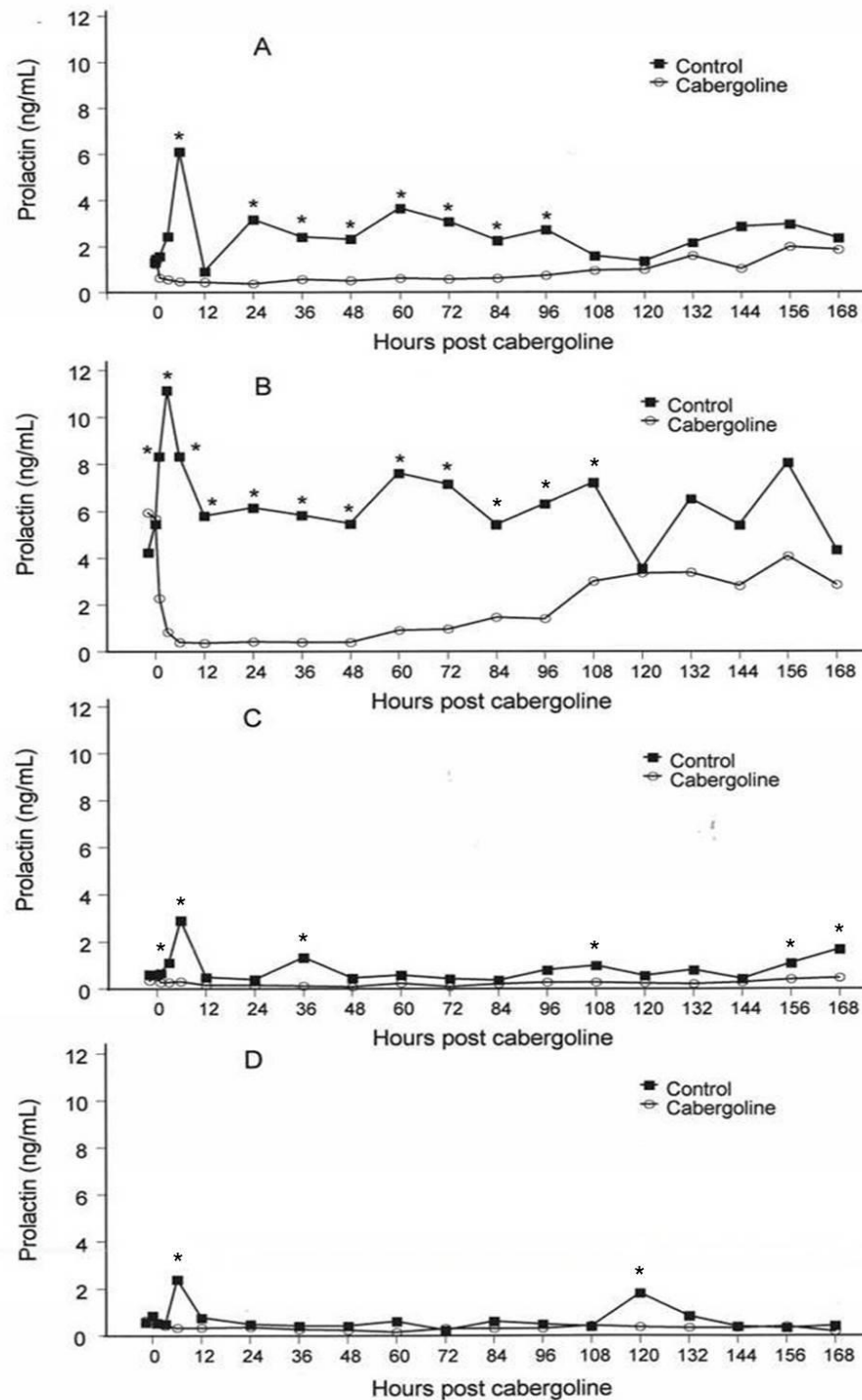


Figure 4.1 Mean plasma prolactin concentrations in mares administered 1.5 mg of cabergoline in diethyl ether-vegetable oil vehicle (Cabergoline) or vehicle only (Control) around the time of the spring equinox (panel A), summer solstice (panel B), autumnal equinox (panel C), and winter solstice (panel D). Asterisks indicate differences ( $P < .05$ ) between groups for specified periods. Pooled standard errors of the mean were 0.69 ng/mL, 1.6 ng/mL, 0.33 ng/mL, and 0.32 ng/mL for panels A-D, respectively.

In Experiment 5 (July), cabergoline suppressed ( $P < .0001$ , Figure 4.2) prolactin in both compounded cabergoline and cabergoline in oil groups with pattern of suppression being virtually identical. Unstimulated prolactin returned to concentrations similar to controls around 132 hours; however, the prolactin response to low dose sulpiride continued to remain greatly suppressed ( $P < .0001$ , Figure 4.3) well after unstimulated levels had returned to normal.

In Experiment 6 (October), cabergoline suppressed ( $P < .01$ , Figure 4.4) prolactin in treated mares for at least 96 hours compared to controls; however, at that time, circulating levels of prolactin in controls dropped remarkably and were indistinguishable from cabergoline-treated mares. The prolactin response to TRH was completely suppressed ( $P < .05$ ) in treated mares through 120 hours (Figure 4.5), but began to recover in subsequent challenges.

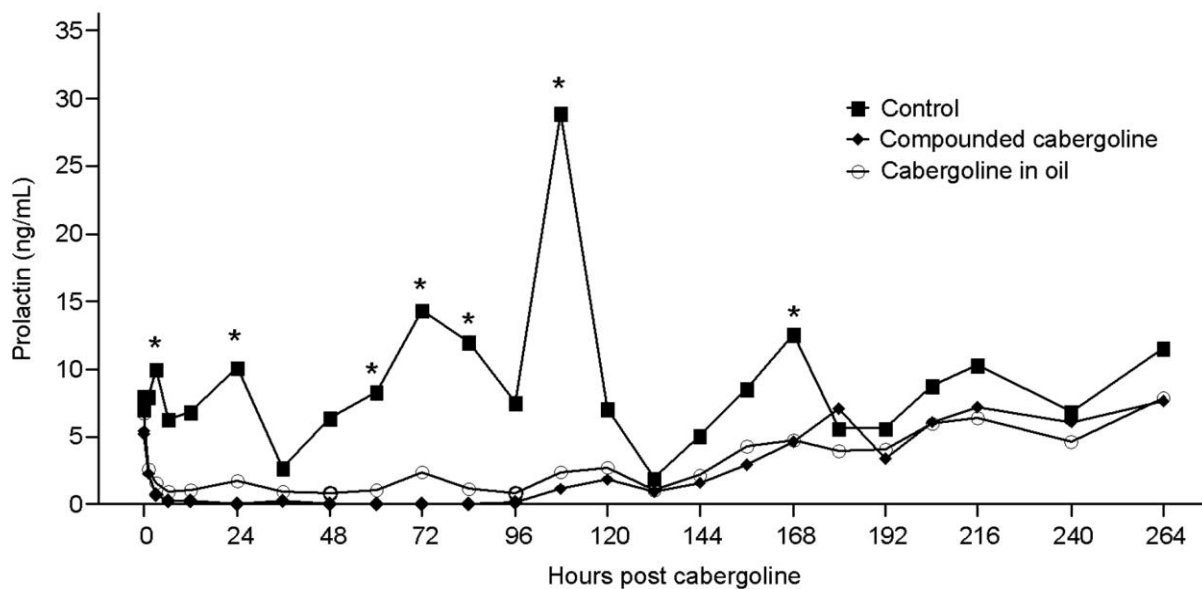


Figure 4.2 Mean plasma prolactin concentrations of mares administered 5 mg compounded cabergoline in a proprietary vehicle (Compounded cabergoline), 5 mg of cabergoline in diethyl ether-vegetable oil vehicle (Cabergoline in oil), or oil only (Control) in July (Experiment 6). Asterisks indicate differences ( $P < .01$ ) between vehicle-treated mares and both groups of cabergoline-treated mares for specified periods. Pooled SEM 5.5 ng/mL.

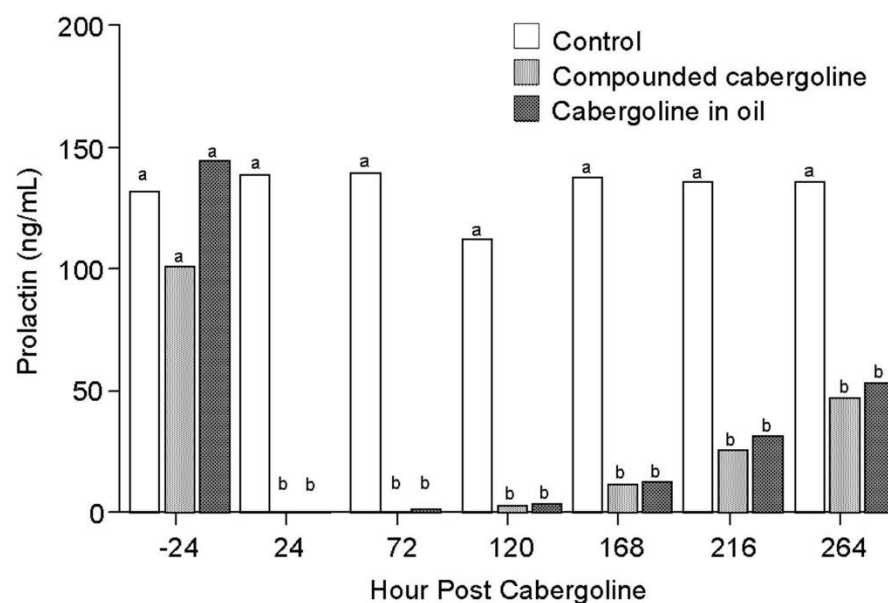


Figure 4.3 Mean plasma prolactin concentrations in response to low dose (.01 mg/kg BW) sulpiride injection (i.v.) administered 24 hours before and then on multiple days after injection of 5 mg cabergoline in proprietary vehicle (Compounded cabergoline), 5 mg cabergoline in diethyl ether-vegetable oil vehicle (Cabergoline in oil), or oil only (Control) in Experiment 6. Data are for the 10-minute samples collected after sulpiride injection (peak values). Means with different letters indicate differences ( $P < .0001$ ) within each specified time period. Pooled SEM was 15 ng/mL.

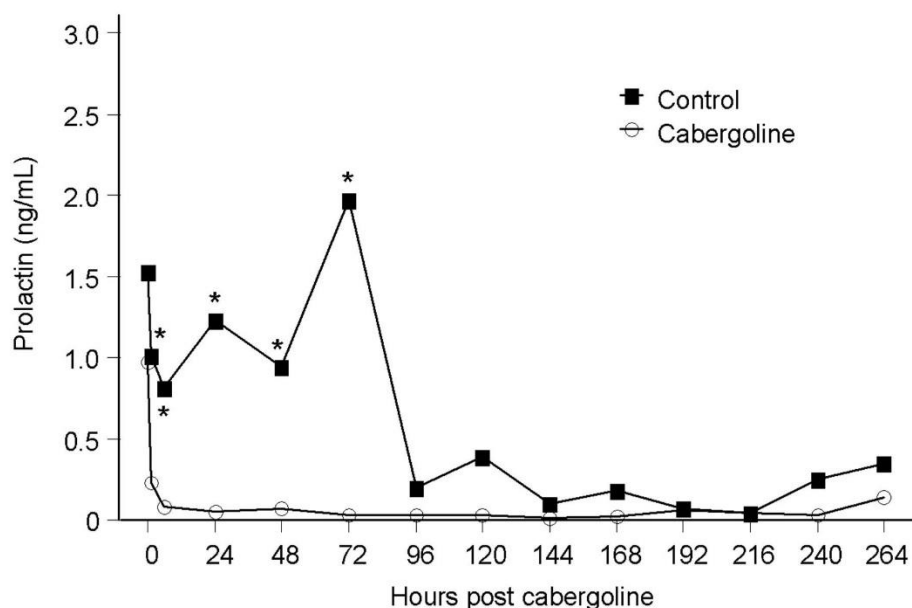


Figure 4.4 Mean plasma prolactin concentrations in mares administered 5 mg of cabergoline in diethyl ether-vegetable oil vehicle (Cabergoline) or vehicle only (Control) in October. Asterisks indicate differences ( $P < .01$ ) between groups for specified periods. Pooled SEM was 0.33 g/mL.

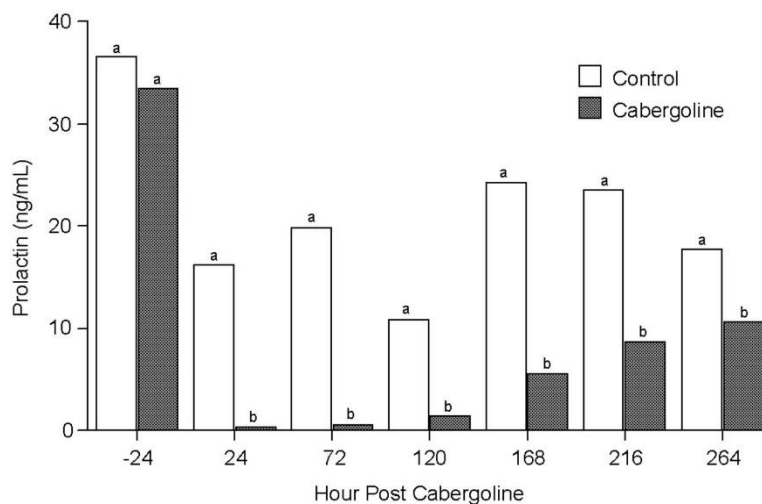


Figure 4.5 Mean plasma prolactin concentrations after treatment with 2 mg/500 kg BW TRH in mares 24 hours before and on multiple time periods after treatment with 5 mg cabergoline in diethyl ether-vegetable oil vehicle (Cabergoline in oil), or oil only (Control). Data are for the 30-minute sample (peak) only. Means with different letters indicate differences ( $P < .05$ ) between groups within each specified time period. Pooled SEM was 15.7 ng/mL.

#### 4.5 Discussion

In the experiments of Hebert et al. [4.8] and Arana Valencia et al. [4.9], both performed in the fall months, 5 mg of cabergoline in the proprietary mixture of oily liquids administered intramuscularly to horses totally suppressed the sulpiride-induced prolactin response for at least 10 days. Although unstimulated (basal, time 0) samples were measured, little change was detected due to the seasonally low prolactin concentrations at that time. Application of the cabergoline suppressive model to test the role of prolactin in vernal transition in spring [4.10] indicated that unstimulated prolactin secretion was totally suppressed after injection of 5 mg of cabergoline for several days, but unlike in the fall experiments, unstimulated prolactin concentrations returned to control levels in 9 days or less. Thus, the experiments presented herein were designed to determine if magnitude and duration of prolactin suppression by administration of cabergoline varied from season to season.

In the first 4 experiments, in lieu of the 5 mg cabergoline treatments administered by Hebert et al. [4.8] and Arana Valencia et al. [4.9], 1.5 mg/500 kg BW of cabergoline was chosen due to limited availability of cabergoline in proprietary vehicle as well as to assess a smaller dose of cabergoline. In both spring and summer, 1.5 mg/500 kg BW cabergoline suppressed prolactin concentrations for a minimum of 96 hours (4 days). In March and July, prolactin returned to values similar to controls by 108 hours and 120 hours, respectively. Thus, suppression of prolactin was similar in duration between spring and summer. Prolactin secretion naturally begins to decline during the fall and reaches its nadir during the winter [4.13-4.15]. Thus, differences in prolactin concentrations between cabergoline- and vehicle-treated mares in fall and winter were not as obvious compared to spring and summer. However, suppression of prolactin was detected in cabergoline-treated mares in the fall and, to a lesser degree, in the winter, similar to what was described by Arana Valencia et al. in fall [4.9]. Some vehicle-treated mares continued to have variable and somewhat elevated prolactin concentrations which made comparisons with cabergoline-treated mares possible during those specific time periods.

In all experiments, a surge of prolactin was observed in vehicle-treated mares in the samples collected 3-6 hours after treatment but not thereafter. This was very consistent from experiment to experiment, indicating it was not due to a single, spurious event. To test whether the oil vehicle, or perhaps the diethyl ether added to the vehicle, was the cause of this unsuspected rise in prolactin, a brief side-trial (unpublished) was conducted testing the individual components versus a no-injection group. It was concluded that neither component was responsible for the prolactin rise. An alternate explanation is that during the 3-6 hour window post treatment on that first day, the horses were removed from the holding chute (in which they had been confined from the start of treatment) and were allowed to graze in the nearby pasture

until the next blood sampling period. Prolactin has been shown to increase consistently after feeding [4.16-4.18], whether it be a full meal of pelleted concentrate or minimal consumption of other feedstuffs [4.17]. Direct gastric infusion of feedstuffs into the stomach by nasogastric intubation did not induce a prolactin response [4.16].

In Experiment 5 (July 2016), 5 mg of cabergoline was chosen to compare the formulation in oil to the dose of compounded cabergoline used by Hebert et al. [4.8] and Arana Valencia et al. [4.9]. Both formulations suppressed prolactin concentrations for 120 hours (5 days) with the pattern of suppression being virtually identical. Again, there was no indication of a seasonal variation. In Experiment 6 (October 2016), 5 mg of cabergoline in oil suppressed circulating prolactin for at least 96 hours (4 days) at which point concentrations of prolactin in vehicle-treated mares decreased rapidly and were indistinguishable from cabergoline-treated mares. This consistent drop in prolactin concentrations in control horses had not been observed in any other experiments, thus further analysis was warranted. Retrospective evaluation of weather conditions during that time revealed that a strong cold front passage, including a thunderstorm followed by a drop in ambient temperature and a shift to northerly winds all occurred coincident with the drop [4.19]. Although no solid evidence has been reported for a temperature effect on prolactin secretion in horses, it is well documented that cold temperatures suppress prolactin secretion in dairy cattle [4.20].

In Experiments 5 and 6, mares were challenged every other day with the dopamine antagonists, sulpiride and TRH, respectively. In both experiments, the prolactin response to either secretagogue was totally absent in cabergoline-treated mares up to 120 hours after treatment. After that, the prolactin response to sulpiride and TRH began to slowly recover, but remained greatly suppressed when compared to vehicle-treated mares. Four to five days into the



experiments, unstimulated prolactin had returned to concentrations similar to vehicle-treated mares, but the prolactin response to either sulpiride or TRH remained suppressed in cabergoline-treated mares up through 11 days. This same pattern of continued suppression of sulpiride-induced prolactin was described by Oberhaus et al. [4.10]: cabergoline-treated mares continued to display greatly suppressed prolactin-responses to sulpiride well in to the early summer months and well after the suppressive effects of cabergoline had waned. This presents an interesting dichotomy of prolactin secretion. It is possible that cabergoline administration results in a down-regulation or desensitization of dopamine receptors on lactotropes, thus preventing those lactotropes from responding to either dopamine agonists or antagonists. But given the same pattern of suppression observed with TRH, a secretagogue that does not interact with dopamine receptors on lactotropes, this does not seem to be the most likely explanation.

Another possible consideration for the dichotomy is the existence of subpopulations of lactotropes which could potentially respond differently to secretagogues and inhibitors and/or could recover faster from inhibition. Subsets of lactotropes have been described for several species [4.21-4.24], including the horse [4.25]. These subsets differ morphologically based on the size of the cell as well as the size and shape of the secretory granules. Christian et al. [4.24] described three morphological subtypes (Type I, II and III) of prolactin-secreting cells in the rat pituitary. By examining exocytosis of prolactin granules, it appeared all three types were inhibited by dopamine, but only Type II and Type III lactotropes were stimulated with TRH or vasoactive intestinal peptide (VIP), another known prolactin secretagogue. Rahmanian et al. [4.25] reported the presence of two morphologically different types of cells in the equine pituitary that both stained positive for prolactin; however, it is not known if both cell types

secrete prolactin similarly in response to secretagogues or inhibitors. Potential differences in functionality of subsets of lactotropes in horses warrants further study.

In summary, treatment with cabergoline suppressed prolactin for at least 4-5 days and duration of prolactin suppression from administration of cabergoline was similar in spring and summer. Given the naturally occurring low concentrations of prolactin in fall and winter, few differences could be detected due to treatment. However, the October data were similar to those reported by Arana Valencia et al. [4.9] for the fall, and could explain the discrepancies between the data of Hebert et al. [4.8] and Arana Valencia et al. [4.9] relative to those of Oberhaus et al. [4.10] in the spring. Low concentrations of prolactin in fall could explain the findings of Hebert et al. [4.8] and Arana Valencia et al. [4.9] relative to those of Oberhaus et al. [4.10]. The formulation of cabergoline in oil provided the same magnitude and duration of prolactin suppression as did the cabergoline in a proprietary vehicle. In fact, other research (unpublished) being conducted at Louisiana State University has described the same duration and magnitude of prolactin suppression from intravenous administration of cabergoline in ethanol, intramuscular administration of cabergoline in oil, and subcutaneous administration of cabergoline in vegetable shortening. Given the potent and long-acting nature of cabergoline, the vehicle may not have an enhance effect on duration of suppression.

In conclusion, no seasonal variation was observed for the duration of suppression or the degree of suppression of prolactin by a fixed dose of cabergoline. However, both sulpiride and TRH-stimulated prolactin continued to be suppressed in cabergoline-treated mares long after unstimulated prolactin concentrations had returned to normal. This apparent dichotomy may indicate at least two subpopulations of lactotropes regulated differentially by dopaminergic input, as has been reported in other species.

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## **CHAPTER 5: FACTORS AFFECTING THE OVARIAN RESPONSE TO A COMBINED ESTRADIOL-SULPIRIDE TREATMENT IN SEASONALLY ANOVULATORY MARES<sup>2</sup>**

### **5.1 Summary**

Twenty-three seasonally anovulatory mares, housed at two separate farms, were treated with 50 mg of estradiol cypionate (ECP) and 3 g of sulpiride in January to study factors that contributed to success of the treatment (response = ovulation within 28 days). Every-other-day blood samples and pretreatment secretagogue challenges were used to characterize prolactin, LH, IGF-I, leptin, and insulin concentrations. Ovaries of each mare were scanned via ultrasound regularly until detection of a 32-35 mm follicle, at which time the mare was artificially inseminated. Prolactin was stimulated in all treated mares and was similar ( $P > 0.05$ ) in responding and non-responding mares. Nine mares, all at the same farm (BH; farm effect,  $P = 0.006$ ), responded with pre-ovulatory sized follicles within 20 days of treatment. Five of the 9 were inseminated and 3 conceived. Retrospective analysis revealed that of the mares responding, body condition score ( $P = 0.03$ ), body weight ( $P = 0.02$ ), plasma concentrations of insulin ( $P = 0.01$ ) and leptin ( $P = 0.09$ ), and pretreatment response of LH to GnRH ( $P = 0.106$ ) were higher in responding than in non-responding mares. In general, factors that differed and may contribute to whether a given mare responds to this ECP-sulpiride protocol were mainly characteristics pointing towards well-nourished mares. Minor nutritional differences between farms likely played a role in the lack of success on the one farm. Also, the LH response to GnRH prior to treatment may be indicative of the subsequent LH response to ECP-sulpiride and hence the ovarian response.

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## 5.2 Introduction

Treatment with exogenous prolactin or an induced increase in endogenous prolactin concentrations can advance the first ovulation in seasonally anovulatory mares [5.1-5.5]. Nequin et al. [5.1] hastened follicular growth in deep anestrous mares with one treatment of ovine prolactin. Thompson et al. [5.2] advanced the date to first ovulation in pony mares in winter by administration of recombinant porcine prolactin, although antibodies to the injected prolactin were eventually detected in those mares. In lieu of exogenous prolactin, various dopaminergic antagonists such as sulpiride [5.3, 5.5-5.7], domperidone [5.4], and fluphenazine [5.1] have been used to stimulate secretion of endogenous prolactin. Sulpiride and domperidone have been used most frequently to stimulate prolactin secretion, a response greatly enhanced by pretreatment with estradiol [5.6-5.8], which was also shown to stimulate luteinizing hormone (LH) in seasonally anovulatory mares [5.6].

In a series of experiments, Mitcham et al. [5.8-5.10] compared prolactin and ovarian responses in mares receiving various doses of estradiol cypionate (ECP) combined with either domperidone or sulpiride, generally at 1.5 or 3.0 g injected 1 to 11 days after ECP injection. Throughout 10 years of experimentation, success rates (percentage of mares ovulating within a specified period of time) have varied from a high of 89% [5.6] down to as low as 50% for the more simplified protocols (treatment with ECP and one injection of sulpiride). In general, mares that respond do so within 28 days. Non-responding mares typically ovulate much later, interspersed with control mares. The question that arises after each experiment is why some mares respond to the selected treatment, whereas others do not. That is, what factor(s), either internal or external, affect(s) whether a mare responds or not?

The aim of the current experiment was to describe and measure as many factors as possible that could potentially affect the response of a given mare. Towards this end, all available anovulatory mares were treated in January with a standard protocol of ECP followed by a single injection of sulpiride, and pretreatment assessments as well as real-time hormonal data were retrospectively used to determine any differences between mares that responded and those that did not respond in the first 28 days. Because previous experiments had not assessed fertility of early ovulations induced by ECP and sulpiride, responding mares were also artificially inseminated with semen from a single stallion once a 32-mm or larger follicle was detected.

### **5.3 Materials and Methods**

Procedures used in these experiments were approved by the Institutional Animal Care and Use Committee of the Louisiana State University Agricultural Center.

#### **5.3.1 Animals**

Mares used in this experiment were housed at one of two Louisiana Agricultural Experiment Station farms: the Central Research Station Horse Unit on the Ben Hur Plantation (BH) and the Reproductive Biology Center (RBC). The two farms were located approximately 9 miles apart south of the Louisiana State University main campus in Baton Rouge. All mares were maintained outdoors throughout the year. They grazed native grass pasture during the warmer months and were supplemented with hay prepared from the same native grasses for *ad libitum* consumption as needed in the fall and winter. In addition to this routine maintenance, mares at the BH farm were also supplemented in winter with 18% protein tubs (Positive Feed, Ltd.; Sealy, TX) and were limit-grazed on winter ryegrass pasture for 1 to 2 hours daily starting in January. Starting in early December, 2015, all non-pregnant mares housed at the two farms were assessed by ultrasonic scanning of the ovaries once a week, and samples of jugular blood were collected



approximately weekly. Anovulation was defined as the absence of any follicle >20 mm, the absence of any corpora lutea, and plasma progesterone concentrations consistently less than 1 ng/mL.

Twenty-three mares were identified that matched the criteria for anovulation: 14 from BH and 9 from RBC. They were all light horse mares, mainly Quarter Horses, Thoroughbreds, and Arabians with unknown reproductive history. Prior to the start of any treatments (first week of January), body weight (BW) and body condition score (BCS; as described by Henneke et al. [5.11]), were recorded for each mare.

### **5.3.2 Pretreatment Assessments, ECP-Sulpiride Treatment, and Blood Sampling**

On January 9, 2016, all mares were challenged intravenously with a combination of gonadotropin releasing hormone (GnRH; 0.5 µg/kg of BW) and sulpiride (racemic mixture; 4 µg/kg of BW) obtained from Sigma-Aldrich, St. Louis, MO. Samples of jugular blood were drawn and 0, 10, 20 and 30 minutes relative to injection to characterize the LH, follicle stimulating hormone (FSH), and prolactin responses. The blood samples were collected into 6-mL evacuated tubes containing sodium heparin as an anticoagulant (Vacurette, Greiner Bio-One, Monroe, NC). The following day (January 10, 2016), all mares received an intramuscular injection in the neck of 50 mg of ECP (BET Pharm, LLC, Lexington, KY). This was considered as day 0 of the experiment.

A comparison of timing of the sulpiride injection component of the standard protocol was overlaid on the experiment, such that approximately half the mares at each farm received sulpiride on day 1 (10 mares, 6 at BH and 4 at RBC) versus the rest that received sulpiride on day 6 (13 mares, 8 at BH and 5 at RBC). Sulpiride injections consisted of 3 g of the racemic mixture of sulpiride suspended in 5 mL of vegetable shortening (Crisco; J.M. Smucker

Company, Orrville, OH) administered subcutaneously in the girth area as previously described by Thompson et al. [5.12]. Jugular blood samples were drawn at 0, 1, 3, 6 and 24 hours relative to injection of sulpiride, and then again every other day until February 28 (day 49). Plasma from these samples was used for the determination of plasma prolactin, leptin, insulin, and insulin-like growth factor-I (IGF-I) concentrations.

An additional jugular blood sample was collected from each mare on the day of ovulation and again for 5 successive days thereafter for determination of plasma LH and progesterone concentrations. For all blood samples collected throughout the experiment, plasma was harvested by centrifugation at 1200 x g for 15 minutes and was stored at -20°C.

### **5.3.3 Ultrasonography and Artificial Insemination**

An ovarian response was defined as the presence of a pre-ovulatory follicle at least 32 mm in diameter within 28 days after sulpiride injection, which either ovulated spontaneously or was induced to ovulate with deslorelin. Ovarian follicular activity was monitored via ultrasonography (Aloka 550V with 5-Mhz linear-array transducer; Hitachi-Aloka, Wallingford, CT) every 3 to 4 days until a follicle 25 mm or greater was detected, after which the mare was scanned daily until the follicle either reached 32 mm or greater, or regressed to < 25 mm. Upon detection of a 32-mm follicle or greater, the mare was artificially inseminated with at least  $500 \times 10^6$  progressively motile sperm from a stallion of proven fertility. After insemination, the mare was administered 0.75 mg deslorelin (BET Pharm, Lexington, KY) intramuscularly to induce ovulation. Inseminated mares were scanned for pregnancy at 12 days after ovulation. If an embryonic vesicle was detected, the mare was scanned again at 16 and 22 days (+/- 1 day). Upon detection of a heartbeat at 22 days or later, the pregnancy was terminated with a luteolytic dose of dinoprost (Lutalyse; Zoetis Inc., Kalamazoo, MI). Some mares ovulated before obtaining a

32-mm follicle, and were not inseminated. Those mares, plus inseminated mares that did not conceive, were scanned every 3 to 4 days to determine their subsequent ovulation.

#### **5.3.4 Hormonal Assays**

Frozen plasma samples were thawed and analyzed for prolactin, FSH, LH, leptin, IGF-I, insulin, and progesterone as appropriate. Prolactin, FSH, LH, leptin and IGF-I were measured with radioimmunoassays previously validated by our laboratory [5.13-5.17]. Intra- and interassay coefficients of variation and levels of detection were 7%, 12% and 0.2 ng/mL for prolactin; 6%, 9%, and 0.2 ng/mL for LH; 7%, 11%, and 1.4 ng/mL for FSH; 5%, 12%, and 8 ng/mL for IGF-I; and 4%, 8%, and 0.8 ng/mL for leptin. Insulin and progesterone concentrations were measured with commercially available kit reagents (ImmuChem Coated Tube <sup>125</sup>I RIA Kit and ImmuChem Double Antibody, <sup>125</sup>I RIA Kit, respectively; MP Biomedicals, Costa Mesa, CA). The intra- and interassay coefficients of variation and assay sensitivities were 5%, 8%, and 0.5 mIU/L for insulin and 5%, 9%, and 0.05 ng/mL for progesterone. Estradiol was measured in acetone-extracted, 0.2 mL samples of plasma with commercially available kit reagents (MP Diagnostics Estradiol 17-β Double Antibody, <sup>125</sup>I RIA Kit); intra- and interassay coefficients of variation and assay sensitivities were 7%, 12% and 1.2 pg/mL.

#### **5.3.5 Statistical Analyses**

Data for single-point dependent variables were analyzed by one-way analysis of variance (ANOVA) using the general linear model of SAS (SAS Instit., Cary, NC). Ovarian response was coded as 0 (no response) or 1 (response) for analysis (see below for treatment groups). Data for dependent variables collected over multiple time points were analyzed by one-way ANOVA with repeated sampling (split-plot design [5.4, 5.18]) with the same software. Retrospective analysis showed that all mares responding with early ovulation were located at one farm (BH). Because

of the lack of success at RBC, analysis as a 2 x 2 factorial (with farm and success as the two factors) was not possible, thus the treatments in the ANOVA were BH responding mares (BHR), BH non-responding mares (BHN), and RBC non-responding mares (RBCN) run individually. Subsequently, although there were no mares responding at RBC to the original ECP-sulpiride treatment, there were 3 mares that displayed more ovarian activity than the rest of the RBC mares, and came close to, but did not achieve, the 32-mm follicle size for breeding. Thus, these mares were lumped together into a fourth treatment group as RBC transitional (RBCT) for analyses of endpoints that might affect success.

All dependent variables were subsequently re-analyzed with the 4 groups in the main analyses (treatments), as well as the timing of sulpiride (1 versus 6 days after ECP) and its interaction with treatment. Time (minutes for the challenges, days for other hormonal characteristics) was included as the repeated factor as appropriate. One-degree of freedom contrasts [5.4, 5.18] were used to assess the effect of farm (BHN+BHR versus RBCN+RBCT) and the difference between responding and non-responding mares at BH (BHN versus BHR) and between the two groupings at RBC (RBCN versus RBCT). The mares within group interaction, which tested treatment in the repeated measures ANOVA, was used as the error term for these comparisons.

## **5.4 Results**

### **5.4.1 Ovarian Responses and Pregnancies**

An ovarian response was defined as the occurrence of a pre-ovulatory follicle at least 32 mm in diameter within 28 days after sulpiride injection, which either ovulated spontaneously or was induced to ovulate with deslorelin. Of the 23 mares treated with ECP and sulpiride, 9 responded with early ovulation (Table 5.1). The average days to ovulation for those mares

Table 5.1. Number of horses treated at each farm that responded with ovulation or a transition-like state.

Farm	Day of Sulpiride Treatment	n	No. Responding	No. Transition-like
RBC	1	4	0 (0%)	1 (25%)
	6	5	0 (0%)	2 (40%)
BH	1	6	5 (83%)	0 (0%)
	6	8	4 (50%)	0 (0%)

Abbreviations: RBC, Reproductive Biology Center; BH, Ben Hur Farm.

responding was 11.7 +/- 1.0, and ranged from 7 to 18 days post-sulpiride injection. There was no difference ( $P > 0.1$ ) in days to ovulation between mares receiving sulpiride on day 1 versus 6 after ECP. Mares not responding to the ECP-sulpiride treatment were scanned weekly until mid-March; no ovulation was detected in any of these mares up to that point.

Of the 9 mares that responded to treatment, all 9 were residents of the BH farm. At RBC, there were 3 mares that grew follicles up to 28 to 30 mm without ovulation. Given that these were the only mares at RBC to show any ovarian response (albeit below threshold), they were re-administered a second injection of sulpiride (same dose as previous) on day 42 (February 22) to determine if perhaps a potentiation had been induced that might lead to a quick response. They indeed responded with ovulatory sized follicles 12, 13, and 16 days after that second sulpiride treatment. Given that these mares stood out from the rest of the mares at RBC, they were placed in a separate group of their own (RBCT) for further analyses.

In the ANOVA for ovarian response, the RBCT mares were included as non-responding, given that they did not ovulate within the original 28-day period. Accordingly, there were only three treatment groups and no orthogonal contrast for RBCN versus RBCT. In that analysis, there was an effect ( $P = 0.0059$ ) of farm but no effect ( $P > 0.1$ ) of day of sulpiride injection nor any interaction with treatment.

Of the 9 BH mares responding to treatment, 5 were artificially inseminated and induced to ovulate with deslorelin, while the remaining 4 ovulated on their own without insemination or deslorelin treatment. Of the 5 that were bred, 3 conceived and carried a pregnancy to 22 days, at which time the pregnancy was terminated. All pregnancies had morphologically normal embryonic vesicles and formation of embryo proper with cardiac activity. The 3 additional mares that received a second sulpiride treatment (RBCT group) were also treated with deslorelin and inseminated, and all 3 became pregnant, again with normal embryonic vesicles and cardiac activity. Thus, the overall pregnancy rate was 6 of 8 mares that were inseminated, or 75%.

#### 5.4.2 Characteristics of Responding and Non-responding Mares

Mean body condition scores, ages, and BW are presented in Table 5.2. The BHR mares had a higher BCS ( $P = 0.06$ ) than the non-responding mares at both farms, but did not differ from RBCT mares. Age and weight were not different overall; however, contrast analyses (Table 5.3) indicated that BHR mares had higher BCS ( $P = 0.03$ ) and weighed more ( $P = 0.02$ ) than BHN mares.

Table 5.2. Mean body condition score (BCS), age, and body weight for horses of the four response groups at the two farms.

Means	Treatment effect				
	BHN	BHR	RBCN	RBCT	P-value
BCS (1-9)	5.2 <sup>a</sup>	6.5 <sup>b</sup>	5.2 <sup>a</sup>	5.3 <sup>a,b</sup>	0.06
Age (yrs)	11	15	11	13	0.23
Weight (kg)	420.6	488.9	478.5	478.0	0.11

Abbreviations: BHN, Ben Hur non-responders; BHR, Ben Hur responders; RBCN, RBC non-responders; RBCT, RBC transition-like.

<sup>a,b</sup>Means within rows with no like superscript differ ( $P < .05$ ).

Table 5.3. One-degree of freedom contrast analyses for body condition score (BCS), age, and body weight between farms and within farms.

	P-value		
	Between Farms	BHN vs. BHR	RBCN vs. RBCT
BCS	0.25	0.03	0.84
Age	0.56	0.11	0.44
Weight	0.30	0.02	0.99

Abbreviations: BHN, Ben Hur non-responders; BHR, Ben Hur responders; RBCN, RBC non-responders; RBCT, RBC transition-like.

Prior to ECP treatment, FSH and prolactin responses to the GnRH and sulpiride challenges were not different ( $P > 0.1$ ; Figure 5.1) across the four groups of mares. Averaged over the three post-GnRH sampling periods, mean concentrations of LH tended to be higher ( $P = 0.105$ ; Figure 5.1) in BHR mares than in BHN mares.

Mean prolactin concentrations in every-other-day samples before and after treatment with ECP plus sulpiride are presented in Figure 5.2. Because there was no effect ( $P > 0.1$ ) of day of sulpiride treatment on ovarian response, the data for all mares were normalized to day of sulpiride treatment (rather than ECP injection). Overall, there was no effect ( $P > 0.1$ ) of farm, no difference between responding and non-responding mares at BH, and no difference between mares in the RBCN and RBCT groups.

Mean plasma LH concentrations before ECP treatment and over the next 18 days are presented in Figure 5.3. A significant effect of treatment ( $P = 0.004$ ) and an interaction ( $P < 0.0001$ ) between response and day were present between responding, non-responding, and RBCT

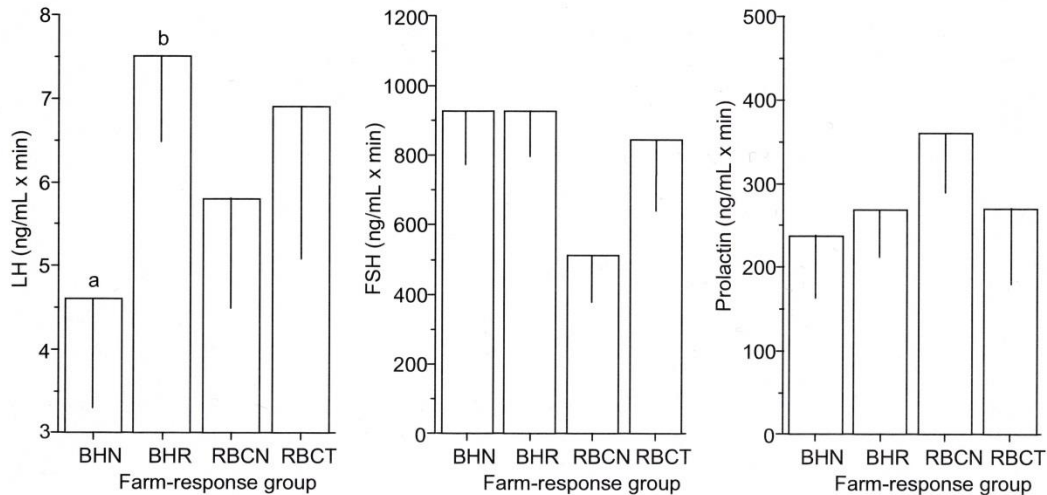


Fig. 5.1. Plasma luteinizing hormone (LH), follicle stimulating hormone (FSH), and prolactin areas under the curve after intravenous injection of gonadotropin releasing hormone and sulpiride 24 hours before ECP injections. The farm-response groups were: mares at the Ben Hur farm that did not respond (BHN), those that did respond (BHR), and mares at the Reproductive Biology Center farm that did not respond (RBCN) and those that showed transitional-like ovarian activity (RBCT). Number of mares per group were 5, 9, 6, and 3, respectively, for the BHN, BHR, RBCN, and RBCT groups. <sup>a,b</sup>Means for LH areas tended to differ ( $P = 0.105$ ). Error variances from the analyses of variance were 0.096, 1218, and 287 for LH, FSH, and prolactin, respectively. The individual standard error of the means are shown by vertical lines within bars.

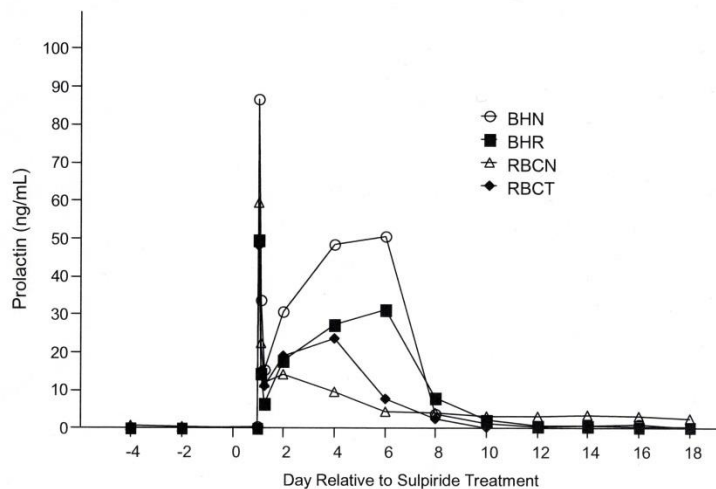


Fig. 5.2. Mean plasma concentrations of prolactin in response to subcutaneous sulpiride injections in January (day 0). The farm-response groups were: mares at the Ben Hur farm that did not respond (BHN), those that did respond (BHR), and mares at the Reproductive Biology Center farm that did not respond (RBCN) and those that showed transitional-like ovarian activity (RBCT). Number of mares per group were 5, 9, 6, and 3, respectively, for the BHN, BHR, RBCN, and RBCT groups. No differences ( $P > 0.05$ ) were detected between any of the four groups of mares. Pooled standard errors of the means from the analysis of variance were 7.38, 5.5, 6.73, and 9.52 ng/mL, respectively.



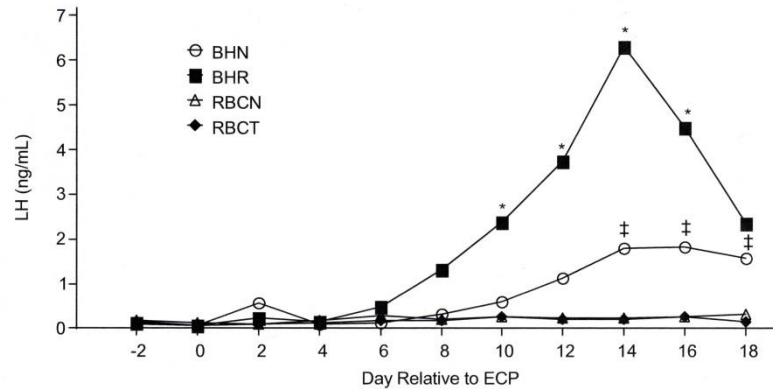


Fig. 5.3. Mean plasma concentrations of LH before ECP treatment (day 0) and over the next 18 days in Ben Hur responding mares (BHR), Ben Hur non-responding mares (BHN), RBC non-responding mares (RBCN), and RBC transitional mares (RBCT). A significant effect of group ( $P = 0.004$ ) and an interaction ( $P < 0.0001$ ) between group and day were present between responding, non-responding, and transitional mares, with LH concentrations being greater in BHR mares on days 10, 12, 14, 16, and 18 post ECP. Luteinizing hormone concentrations were also greater ( $P < 0.05$ ) in BHN mares than in RBCN or RBCT mares on days 14, 16 and 18 post ECP treatment. Pooled standard errors of the means from the analysis of variance were 0.54, 0.41, 0.5, and 0.7 ng/mL, respectively. Asterisks (\*) indicate differences between BHR and all other groups; double daggers (‡) indicate differences between BHN and all other groups.

mares, with LH concentrations being greatest in BHR mares on days 10, 12, 14, 16 and 18 after ECP. Luteinizing hormone concentrations were also greater ( $P < 0.05$ ) in BHN mares than in RBCN or RBCT mares on days 14, 16 and 18 post ECP treatment.

Plasma estradiol concentrations after treatment with ECP were measured in BH mares only, given that no RBC mare ovulated within 28 days. There was no difference ( $P > 0.1$ ) in plasma estradiol concentrations between the responding and non-responding mares at BH (Figure 5.4). Plasma estradiol peaked 1 to 2 days post ECP treatment and started a slow decline through day 16 after ECP.

Means for plasma IGF-I, leptin, and insulin concentrations are presented in Table 5.4. Concentrations of IGF-I did not differ ( $P > 0.1$ ) among the four groups, nor was there any effect

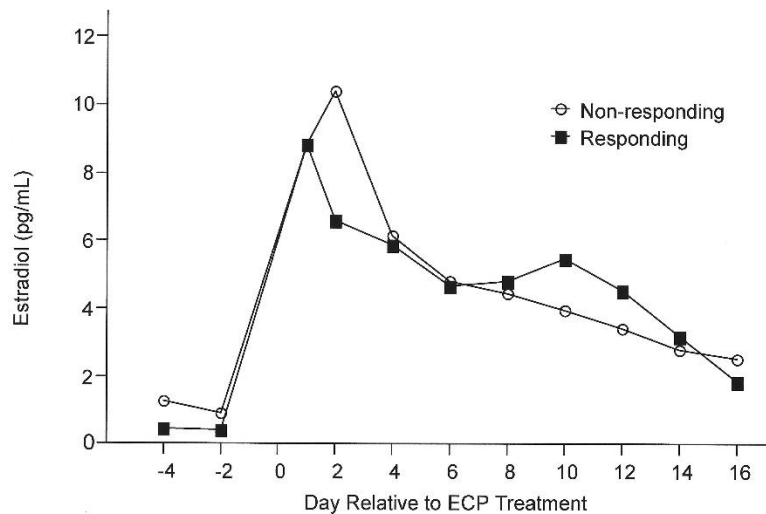


Fig. 5.4. Mean plasma estradiol concentrations in mares at the Ben Hur farm after treatment with 50 mg of ECP on day 0. Estradiol concentrations in mares at the Reproductive Biology Center farm were not assessed given that no mare responded on that farm with a 32-mm or greater follicle within 28 days. Pooled standard error of the mean from the analysis of variance was 2.13 pg/mL.

of farm. There was an effect of treatment ( $P = 0.01$ ) on leptin concentrations as well as an interaction between treatment and day ( $P < 0.0001$ ). Leptin concentrations were highest in RBCT mares; however, the difference was due primarily to two of the mares in particular with higher than normal leptin concentrations. Contrast analyses (Table 5.5) revealed that leptin

Table 5.4. Mean plasma concentrations of IGF-I, leptin and insulin.

Mean ( $\pm$ SEM) plasma concentration, (ng/mL)					Treatment effect	Interaction with day
Hormone	BHN	BHR	RBCN	RBCT	P-value	
IGF-I	138.4 $\pm$ 4.8	172.2 $\pm$ 3.5	84.97 $\pm$ 4.3	127.25 $\pm$ 6.0	0.16	0.91
Leptin	0.24 $\pm$ .04 <sup>a</sup>	0.87 $\pm$ .03 <sup>b</sup>	0.40 $\pm$ .04 <sup>a,b</sup>	1.85 $\pm$ .05 <sup>c</sup>	0.01	< 0.0001
Insulin	21.73 $\pm$ 2.1 <sup>a</sup>	36.62 $\pm$ 1.6 <sup>b</sup>	30.18 $\pm$ 1.9 <sup>a,b</sup>	37.08 $\pm$ 2.7 <sup>b</sup>	0.05	0.33

Abbreviations: BHN, Ben Hur non-responders; BHR, Ben Hur responders; RBCN, RBC non-responders; RBCT, RBC transition-like.

<sup>a-c</sup>Means within rows with no like superscript differ ( $P < 0.05$ ).

concentrations were higher ( $P = 0.09$ ) in the BHR mares than in the BHN mares, and were higher ( $P = 0.003$ ) in the RBCT mares than in the RBCN mares. There was also an effect of treatment on insulin concentrations ( $P = 0.05$ ), which were highest in RBCT and in BHR mares. Contrast analyses (Table 5.5) showed that concentrations of insulin were higher ( $P = 0.01$ ) in BHR mares than in BHN mares.

Of the 9 mares responding at the BH farm, 5 were induced to ovulate with deslorelin while the remaining 4 ovulated normally without deslorelin. All mares that responded with ovulation experienced a normal rise in progesterone and decline in LH immediately following ovulation (Figure 5.5). There was no difference ( $P > 0.1$ ) between mares that were induced to ovulate with deslorelin and inseminated and mares that ovulated spontaneously and were not inseminated, indicating that addition of deslorelin provided no advantage over no treatment with deslorelin.

Table 5.5. One-degree of freedom contrast analyses for plasma IGF-I, leptin, and insulin concentrations between farms and within farms.

Hormone	P-value for contrast		
	Between Farms	BHN vs. BHR	RBCN vs. RBCT
IGF-I	0.14	0.42	0.40
Leptin	0.06	0.09	0.003
Insulin	0.29	0.01	0.29

Abbreviations: BHN, Ben Hur nonresponders; BHR, Ben Hur responders; IGF-I, insulin-like growth factor-I; RBCN, RBC nonresponders; RBCT, RBC transition like.

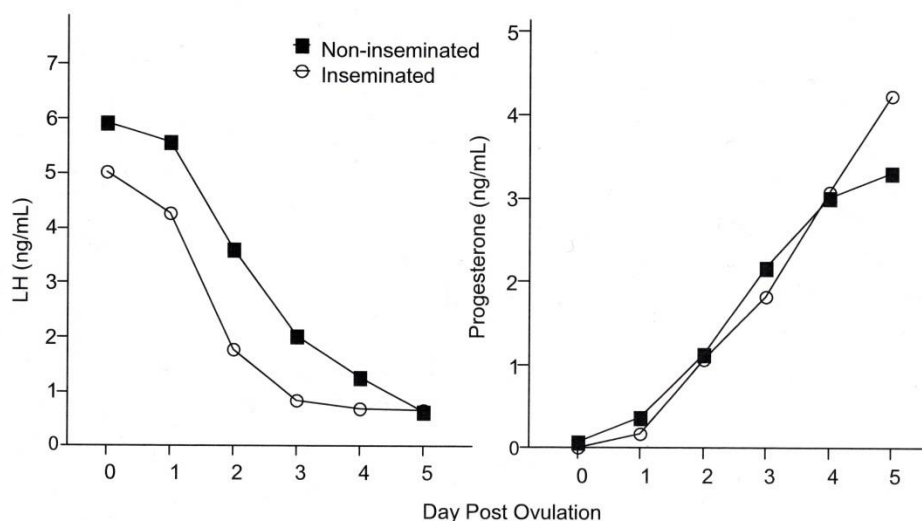


Fig. 5.5. Mean plasma LH and progesterone concentrations on day of ovulation (day 0) and for 5 successive days after. No differences existed ( $P > 0.1$ ) between mares that were induced to ovulate with deslorelin and inseminated and mares that ovulated spontaneously and were not inseminated. Pooled standard errors of the means from the analysis of variance were 1.7 ng/mL for LH and 0.99 ng/mL for progesterone.

## 5.5 Discussion

The use of dopamine antagonist protocols to induce early ovulation in mares has met with varying results. Some mares respond similarly from season to season, while others either never respond or will respond one season, but not the next (E.L. Oberhaus, *personal observation*). The aim of this study was to retrospectively analyze factors that might contribute to or prevent a mare from responding to ECP and sulpiride with early follicular activity and ovulation. Also, while dopamine antagonist protocols to induce ovulation have repeatedly been studied in our lab and by others, it has not been determined whether ovulations induced by ECP-sulpiride are indeed fertile and whether the uterus would support early pregnancy.

In this study, all mares were treated with ECP and a single subcutaneous injection of sulpiride in vegetable shortening. Simultaneous controls (no treatment) were not included for two reasons: 1) in order to analyze factors affecting success, we wanted as many mares as

possible involved in the treatment to maximize the number and potential diversity of the group, and 2) after 33 years of similar experimentation [5.2, 5.6-5.10, 5.19-5.23, 5.25], mares at the LSU AgCenter farms that have been selected as anovulatory by the criteria described herein and have served as controls have ovulated spontaneously in January or February only infrequently (between 0 and 11%, depending on year), and typically begin ovulating in mid-march or later, with an overall mean date of April 1. Experimental reduction in BCS, as described by Gentry et al [5.25], extended the period of anovulation well into late April and May. In contrast to mares selected as anovulatory, a certain percentage of mares on these same farms will have significant follicular activity throughout the winter, and a few will continue to experience regular estrous cycles.

Ten mares received sulpiride 1 day after ECP while 13 received sulpiride 6 days after ECP. Mitcham et al. [5.8] observed a tendency for prolactin response to be higher in mares receiving domperidone 1 day after ECP versus 6 days after ECP; however, in the present study, day of sulpiride treatment did not affect the degree to which prolactin concentrations were increased. Kelley et al [5.6] did not start sulpiride treatment until 10 days after the initiation of estradiol injections. It is apparent from the results of all of our experiments combined that considerable leeway exists for the period between ECP injection and sulpiride injection. Whether simultaneous ECP and sulpiride injections would produce similar success rates needs to be tested in the field.

Ovarian success, as defined by ovulation within 28 days post sulpiride treatment, was relatively low in this experiment (9 of 23 mares, or 39%), whereas all 9 responding mares were housed at one farm. Considering only mares at the BH farm, success was 9 of 14 mares, or 64%. Success rates of 50 to 89% have been achieved in previous experiments [5.6-5.10], depending

upon the specific protocol used. The 89% success rate of Kelley et al. [5.6] was based on every-other-day injections of estradiol benzoate followed by daily sulpiride injections started 11 days later. Attempts to simplify that protocol into a single or double injection regimen is what has led to the lower percentages of success. In the last experiment reported by Mitcham [5.10], a single injection of ECP followed by three injections of sulpiride 5 days apart resulted in a success rate of 77%. Reducing the number of sulpiride injections to two, 5 days apart, produced a success rate of 63% (E.L. Oberhaus, *personal observation*). For the present experiment, the number of sulpiride injections was reduced to one in an effort to simplify the protocol even further.

Pre-trial assessments of the LH, FSH, and prolactin responses to a standardized GnRH-sulpiride challenge revealed no predictive information regarding FSH or prolactin responses prior to ECP-sulpiride treatment. There was a tendency for a difference in LH response. The tendency for a greater LH response to GnRH in BHR compared to BHN and RBCN may indicate that the adenohypophyses of mares that would go on to respond to treatment were already more competent in terms of LH synthesis and release, albeit low at that time of year. Given that no mares had ovarian follicle(s) greater than 15 mm before the experiment started, all mares were considered to be in a relatively “deep” anestrus-anovulatory state. This tendency of higher LH response was evident in the responses to ECP injection, with the BHR mares having a much greater rise in LH concentrations 10 to 18 days after ECP relative to BHN mares. The almost total lack of LH response to ECP in the mares at RBC may account for the lack (or relative lack) of ovarian stimulation.

Prolactin was stimulated in all mares for approximately 7 days by the injection of 3 g of sulpiride in vegetable shortening. The response to sulpiride did not differ between mares that ovulated early and mares that did not, nor did it differ between the two farms. An important point

is that the responses were robust, with little variation, in contrast to what was often observed with domperidone injections in previous experiments [5.7-5.9]. Thus, if there is indeed a threshold level of prolactin concentrations that must be achieved for ovarian success, then it is likely that all mares at both farms reached that threshold, whereas only mares at BH had sufficient LH response to ECP to reach an ovulatory state. Previous injections of ECP alone, with no dopaminergic antagonist to stimulate prolactin secretion, did not produce successful ovarian responses [5.10]. In all our previous experiments with the estrogen-dopaminergic antagonist combination, it has been consistent that both the prolactin response and the LH response to estrogen must occur to some extent for the ovaries to respond. Failure of either results in zero success.

It is well documented that nutrition and body condition play an important role in reproduction [5.24-5.27]. Gentry et al. [5.25] demonstrated that low BCS of 3.0 to 3.5, produced by nutrient restriction, resulted in low plasma leptin and IGF-I concentrations, low unstimulated prolactin concentrations, a low LH response to GnRH, and an extended seasonal period of anovulation with minimal follicular activity. Similarly, Guillaume et al. [5.26] observed an increase in winter ovarian inactivity in feed-restricted pony mares, although to a lesser degree than observed by Gentry et al. [5.25]. Interestingly, the prolactin response to sulpiride reported by Gentry et al. [5.25] did not differ between high and low BCS mares, much like that observed in the present experiment. Albeit speculation, parallels can be seen between the differences observed herein between responding and non-responding mares, and the results of Gentry et al. [5.25]: low BW and BCS, low plasma leptin, low LH response to GnRH, and in this experiment, low insulin concentrations.

In this experiment, plasma IGF-I concentrations did not differ between groups of mares, but leptin and insulin were higher in the BHR than in the BHN mares even prior to treatment with ECP and sulpiride. These hormones are generally thought of as indicators of nutritional status, with higher concentrations associated with better nutrition. For mares housed at the BH farm, the differences would be consistent with that concept. For the RBC mares, the trends are in the same direction, but did not meet statistical muster. Due to different winter management schemes at the two farms, differences in nutritional availability were present, even though all mares were housed on pasture without any grain supplementation. Retrospective investigation revealed that the mares at the BH farm had access to round bales of Alicia bermuda grass hay, winter ryegrass pasture, and *ad libitum* protein tubs (primarily cottonseed and soybean meals, molasses, and added vitamin A). Mares at the RBC farm had access to round bales of coastal bermuda and rye grass hay, but did not have access to winter ryegrass or protein supplementation. These differences in potential nutrient intake may have contributed to the failure of RBC mares to respond to the initial treatment.

The three RBC mares that were considered transitional due to significant follicular activity (relative to the other nonresponding mares) with no ovulation had the highest leptin concentrations of all mares in the four groups. Of those three mares, two had leptin concentrations ranging from 2 to 5.5 ng/mL compared to < 2 ng/mL for most other mares at both farms. It is possible that these mares were hyperleptinemic as a result of insulin insensitivity [5.16, 5.28], although no direct assessment of insulin sensitivity was performed. This is somewhat contradictory to the findings of Ferreira-Dias et al. [5.27] where mares that continued to cycle through the winter displayed leptin concentrations as high as 8 ng/mL with no reported perturbations in cyclicity. Hyperleptinemia is typically associated with hyperinsulinemia, due to



compensated insulin resistance [5.16, 5.28]. However, the mean insulin concentrations in these three mares were not outstanding relative to the other mares at both farms. Insulin insensitivity per se is known to be associated with perturbations in estrous cyclicity [5.29, 5.30], thus studies involving the use of sulpiride in these mares deserve further research. The fact that these three mares responded so consistently and quickly to the second sulpiride injection on February 22 may indicate that the first ECP-sulpiride treatment established conditions within the ovaries that potentiated the response to subsequent elevated prolactin concentrations.

The effect of ECP and sulpiride on LH concentrations seems to be somewhat complex. Clearly, estradiol has a stimulatory effect on LH secretion, and an increase in LH has not been observed in mares receiving a dopamine antagonist alone [5.6-5.10]. Garcia and Ginther [5.31] administered 1 mg of estradiol daily to ovariectomized pony mares in February and observed an increase in LH, but not until 6 days after initiation of treatment. Similarly, Kelley et al. [5.6] and Mitcham [5.10] administered 11 mg estradiol benzoate and 150 mg ECP, respectively, to seasonally anovulatory mares and did not observe an increase in LH until after treatment with sulpiride 6 to 11 days after estrogen treatment. Moreover, Mitcham [5.10] administered sulpiride or domperidone in biodegradable microparticles to mares simultaneously (same morning) as the ECP injection and observed no increase in plasma LH concentrations over the next 21 days. The question arises as to whether treatment with sulpiride after ECP enhances the LH response. In the present study, an increase in LH was not observed until 10 days after ECP treatment. At that point, concentrations of LH rose to levels similar to those seen around a typical, breeding season ovulation, and appeared to coincide with late stage follicular growth and ovulation in those (BHR) mares. Plasma estradiol in responding and non-responding mares at BH peaked one and two days, respectively, after ECP treatment and then began to decline. Four of the 9 responding

mares exhibited a decline in circulating estradiol followed by a subsequent rise around the time of each mare's respective ovulation; therefore, it is possible that LH is being stimulated by endogenous follicular estradiol as well as the exogenous ECP.

Mari et al. [5.5] and Panzani et al. [5.32] assessed fertility following sulpiride treatment in anestrus mares. First ovulation of the season was advanced in sulpiride-treated mares and pregnancy rates on the first cycle were 40% (4/10) in one study [5.5] and 63.6% (14/22) in another [5.32]. In addition, Mari et al. [5.5] reported normal foalings in all pregnant mares. Throughout the series of experiments with estrogen pretreatment coupled with antidopaminergic agents that we have completed [5.6-5.10], this is the first time to test the actual fertility of the ovulations induced by treatment. Although minimal in number, the percentage of mares becoming pregnant after insemination is certainly within the expected range for mares bred on one estrous cycle in spring and summer. More extensive studies are needed to confirm whether the fertility of these induced ovulations is indeed equivalent to later breedings. However, these data are encouraging for breeders who want to get their open mares pregnant early in the year.

In conclusion, treatment with 50 mg of ECP followed by 3 g of +/- sulpiride in vegetable shortening 1 or 6 days later elevated plasma prolactin concentrations in all mares for 6 to 8 days. Given the robust responses across all mares, prolactin response could not be a factor in determining whether a mare responded within 28 days or not (success). Assessments of plasma FSH and prolactin responses to low-dose sulpiride and GnRH challenges provided no predictive information as to future success. The tendency for higher LH response to GnRH in responding mares prior to treatment may be indicative of the subsequent LH response and eventually the ovarian response to ECP-sulpiride. Success was influenced by body condition and likely nutritional status as indicated by higher plasma leptin and insulin concentrations in the

responding mares. We suspect that the likelihood of a seasonally anovulatory mare responding to the treatment protocol used herein is inversely correlated to the “depth” of the anestrous state. That depth of anestrus is known to be influenced by nutritional status as well as proximity to the seasonal transition period, as we [5.25] and others [5.33] have suggested in previous reports for mares treated with only dopaminergic antagonists. Finally, from the limited inseminations performed with these mares, we suspect that these induced ovulations are indeed fertile and can produce at least 22-day embryos with heartbeats. Future research would be needed to better clarify the actual fertility rate and to confirm that the pregnancies can be carried to term.

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## **CHAPTER 6: EFFECTS OF COMBINED ESTRADIOL-SULPIRIDE TREATMENT AND FOLLICLE ABLATION ON VERNAL TRANSITION IN MARES: EVALUATION OF PLASMA AND FOLLICULAR FLUID HORMONES AND LH RECEPTOR GENE EXPRESSION**

### **6.1 Summary**

This experiment was designed to assess the hormonal production, secretory aspects, and changes in LH receptor gene expression of early-induced ovulatory-sized follicles relative to the first ovulatory sized follicles occurring naturally in the spring. Seasonally anovulatory mares were treated on January 21 with 1) 50 mg estradiol cypionate (ECP,  $n = 8$ ) alone or 2) with ECP on January 21 followed by 2 sulpiride injections (3 g, s.q., in vegetable shortening,  $n = 8$ ) 5 and 12 days later. Half of each group also received complete follicle ablation via transvaginal aspiration prior to ECP treatment. Ovaries were scanned via ultrasonography regularly until detection of a 32-35 mm follicle; follicular fluid was then recovered via aspiration and analyzed for prolactin, estradiol and progesterone concentrations. Blood was collected every 4 days to characterize plasma prolactin, LH, FSH, and estradiol concentrations. Also, on the day of aspiration and for 6 more days, jugular blood was collected to characterize LH and progesterone concentrations. Mean date to first 35-mm follicle was advanced ( $P < .05$ ) in sulpiride-treated mares: 5 of the 8 (63%) responded within 28 days of the first sulpiride treatment. Complete follicle ablation did not affect ( $P > .1$ ) the ovarian response. Plasma prolactin was stimulated ( $P < .0001$ ) in ECP-sulpiride treated mares for 16 days. There was no difference ( $P > .1$ ) in plasma prolactin in mares that responded with a large follicle versus mares that did not. Estradiol treatment stimulated plasma LH in all mares ( $P < .05$ ) and ablation did not affect ( $P > .1$ ) LH response to ECP. Plasma LH was higher ( $P < .05$ ) in treated mares that responded compared to mares that did not respond on days 8 and 12 after sulpiride treatment. In plasma samples collected daily after aspiration, both LH and progesterone were higher ( $P < .01$ ) in mares that

were treated with ECP-sulpiride and were not ablated compared to ECP-only treated mares (both ablated and not) and to ECP-sulpiride treated mares that were ablated. Plasma estradiol was similar ( $P > .1$ ) in all mares on day of aspiration. There was a tendency for an increase in follicular fluid prolactin in sulpiride-treated mares. There was no effect ( $P > .1$ ) of treatment or ablation on follicular fluid concentrations of estradiol, progesterone, leptin, or IGF-1, or on LH receptor gene expression. In conclusion, combined ECP-sulpiride treatment stimulated circulating prolactin and hastened the date to first pre-ovulatory follicle, but degree of prolactin response was not a predictor of ovarian response. Sulpiride-treated mares that were not ablated had elevated plasma progesterone and LH after aspiration. Ablation may inhibit maturity of the first pre-ovulatory follicle based on decreased progesterone and LH production, but does not inhibit growth and development of the first pre-ovulatory follicle.

## **6.2 Introduction**

Stimulation of prolactin, either through administration of exogenous prolactin or via administration of dopaminergic antagonists, has been shown to stimulate ovarian follicular growth and hasten the date to first ovulation in seasonally anovulatory mares [6.1-6.5]. Nequin et al. [6.1] hastened follicular growth in seasonally anestrous mares with one treatment of ovine prolactin. Similarly, Thompson et al. [6.2] administered recombinant porcine prolactin to winter anestrous pony mares and advanced the first ovulation of year. Sulpiride and domperidone have been used most frequently to stimulate prolactin secretion, a response greatly enhanced by pretreatment with estradiol [6.6-6.11], which was also shown to stimulate luteinizing hormone (LH) in seasonally anovulatory mares [6.6, 6.11]

The exact mechanism by which prolactin stimulates follicular growth in seasonally anovulatory mares has yet to be identified. Receptors for prolactin have been localized on



equine ovarian follicular cells [6.12] as well as luteal cells [6.13], which is an indication that prolactin can exert its action directly on the ovarian follicle. Prolactin has an obligatory role in ovarian function in several species, such as formation and maintenance of the corpus luteum (CL; [6.14, 6.15]). It has also been suggested that prolactin is responsible for inducing functional LH receptors on granulosa cells and CL of rats as well as sustaining progesterone secretion from the CL [6.16-6.18]. Richards and Williams [6.16] and Holt et al. [6.17] observed an enhanceive effect of prolactin on LH receptor content and progesterone production, but not a direct effect of prolactin alone. Conversely, Bjurulf et al. [6.18] observed a ten-fold increase in LH receptor messenger mRNA in luteal cells of prolactin-treated rats as well as an increase in circulating progesterone concentrations when compared to controls. Furthermore, a marked decrease in LH receptor mRNA was detected in prolactin receptor null mutant mice [6.19]. This effect of prolactin on LH receptors and receptor mRNA in females is directly analogous to the complete requirement for prolactin for spermatogenesis in the male hamster [6.20, 6.21], which was shown to be mediated by prolactin's necessity for LH receptors on hamster Leydig cells [6.22].

In the mare, a local role for prolactin in the ovary has been proposed due to the remarkable and rapid growth of ovarian follicles in response to either exogenous prolactin or indirectly through treatment with dopaminergic antagonists in the winter. Exactly how exogenous or endogenously stimulated prolactin facilitates early follicular growth has yet to be determined. Therefore, the aim of this study was to induce early follicular growth in seasonally anestrus mares with treatment of ECP and subsequently sulpiride as described by Mitcham et al. [6.10] and Oberhaus et al. [6.11] and assess changes in ovarian follicle hormone production and LH receptor content. The effect of complete follicle ablation prior to treatment was also

assessed to determine if the induction of a new follicular wave had an effect on the ovarian response to treatment.

## **6.3 Materials and Methods**

Procedures used in these experiments were approved by the Institutional Animal Care and Use Committee of the Louisiana State University Agricultural Center.

### **6.3.1 Animals and Treatments**

Mares used in this experiment were housed at one of two Louisiana Agricultural Experiment Station farms: the Central Research Station Horse Unit on the Ben Hur Plantation (BH) south of the LSU campus and the Reproductive Biology Center (RBC) located in St. Gabriel, Louisiana. The two farms were located approximately 9 miles apart south of the Louisiana State University main campus in Baton Rouge. All mares were maintained outdoors throughout the year. They grazed native grass pasture during the warmer months and were supplemented with hay prepared from the same native grasses for ad libitum consumption as needed in the fall and winter.

Starting on December 29, 2014, all non-pregnant mares housed at the two farms were assessed for 3 weeks by weekly ultrasonic scanning of the ovaries sampling of jugular blood. Anovulation was defined as absence of a follicle > 20 mm in diameter on either ovary, absence of any corpora lutea, and plasma progesterone concentrations consistently < 1 ng/mL.

Sixteen light horse, anovulatory mares were identified (8 at each farm) and were allotted into two similar groups based on age (7 to 25 years old) and body condition score (4 to 7; [6.23]). The groups were then randomly assigned to treatment (n = 8) or control (n = 8). Half of each group was subjected to complete follicle ablation 5 days prior to sulpiride treatment.

On January 21, 2015, all mares received 50 mg estradiol cypionate (ECP, BET Pharm, LLC, Lexington, KY), intramuscularly. Five days later, on January 26, mares allotted to the combined treatment group (n = 8) received 3 g of sulpiride (racemic mixture; Sigma-Aldrich, St. Louis, MO) dissolved in 5 mL of vegetable shortening (Crisco; J.M. Smucker Company, Orrville, OH) administered subcutaneously in the girth area as previously described by Thompson et al. [6.24]. Control mares (n = 8) received 5 mL vegetable shortening only in the girth area. All mares received a second sulpiride or control treatment of the same nature 7 days later on February 2.

### **6.3.2 Blood Sampling**

On January 21, 2016, just prior to treatment with ECP, jugular blood samples were collected from each mare into 6-mL evacuated tubes containing sodium heparin as an anticoagulant (Vacuette, Greiner Bio-One, Monroe, NC). On January 26 (day 0), samples were drawn at 0 min, 1, 3, 6, 12 and 24 hours relative to treatment with sulpiride or vehicle and continued every four days until April 16 (day 80) to determine circulating plasma prolactin, LH, FSH, and estradiol concentrations. Additionally, on the day of aspiration and for five successive days after, a single blood sample was drawn to determine circulating concentrations of LH and progesterone. Plasma was harvested from all samples in the experiment by centrifugation at 1200 x g for 15 minutes and was stored at -20°C.

### **6.3.3 Ultrasonography and Transvaginal Follicle Aspiration**

On the day of ECP treatment (day -5), just prior to treatment, half of the mares in each group received a complete follicle ablation which involved aspiration of all visible follicles on each ovary prior to any treatment via the aspiration procedure described below. After treatment with ECP, ovarian activity was monitored via ultrasonography (Aloka 550V with 5-Mhz linear-

array transducer; Hitachi-Aloka, Wallingford, CT) every 3 to 4 days until a follicle(s) >25 mm emerged. Upon detection of a follicle >25 mm, the mare was scanned daily until the follicle either reached at least 35 mm or regressed to <25 mm. Once a 35-mm follicle was observed, the follicle was aspirated.

For aspirations, including those during complete follicle ablation, each mare was administered intravenous detomidine (.01 mg/kg BW; Dormosedan, Zoetis, Parsippany-Troy Hills, NJ) for sedation and N-butylscopolammonium bromide (.25 mg/kg BW; Buscopan, Boehringer Ingelheim Vetmedica, Inc, St. Joseph, MO) for rectal relaxation. Additional amounts of these medications were administered as needed. A 5 MHz curvilinear probe housed in hard plastic casing was used for visualization and aspiration. The follicle was aspirated with a 12-gauge double lumen needle attached to a vacuum-pump at a pressure of -150 mmHg. The probe was inserted transvaginally and placed directly against the vaginal wall. The ovary was then manipulated transrectally such that the follicle was placed next to the end of the ultrasound probe. A second technician advanced the needle through the vaginal wall and into the follicle. The follicle was immediately evacuated of follicular fluid in to a sterile 50 mL conical tube; the fluid was stored for later analysis of prolactin, estradiol, progesterone, IGF-1 and leptin. The follicle was then flushed several times with Dulbecco's phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, MO) supplemented with 10 U/mL heparin and 1.5% bovine calf serum warmed to 38°C and the flushing fluid was evacuated into a sterile bottle. Upon collection of follicle contents, cells were filtered to remove blood contamination, rinsed with PBS, and manually pipetted into cryovials. Cells were centrifuged at 700 x g for 15 min and pellets resuspended in RNAlater (Sigma-Aldrich, St. Louis, MO) and stored at -80°C until analysis.

#### **6.3.4 Radioimmunoassay**

Frozen plasma samples were thawed and analyzed for prolactin, LH, FSH, progesterone and estradiol as appropriate. Follicular fluid samples were analyzed for prolactin, progesterone, estradiol, IGF-1 and leptin concentrations. Prolactin, LH, FSH, IGF-I, and leptin were measured by radioimmunoassay in assays previously validated by our laboratory [6.25-6.29]. Intra- and interassay coefficients of variation and levels of detection were 7%, 12% and 0.2 ng/mL for prolactin; 6%, 9%, and 0.2 ng/mL for LH; and 7%, 11%, and 1.4 ng/mL for FSH; 5%, 12%, and 8 ng/mL for IGF-I; and 4%, 8%, and 0.8 ng/mL for leptin. Progesterone and ether-extracted estradiol were analyzed using commercially available kit reagents (ImmuChem Progesterone Double Antibody, 125I RIA Kit and 17 $\beta$ -Estradiol (E2) Double Antibody RIA Kit, MP Biomedicals, Inc, Costa Mesa, CA). For estradiol determination in follicular fluid, samples were diluted to a final concentration of 1:5000 before extraction with extracted with diethyl ether.

#### **6.3.5 RNA Extraction and Q-PCR**

Total RNA from cell aspirates was extracted using the RNeasy Micro Kit (Qiagen, Valencia, CA) per manufacturer's instructions. Complementary DNA (cDNA) was generated using iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA). For each reverse transcription reaction, total RNA was added to 4  $\mu$ L reverse transcription supermix and nuclease-free water for a total volume of 20  $\mu$ l per reaction. Reverse transcription was performed following the manufacturer's recommendations with 5 min at 25°C, 20 min at 46°C, and 1 min at 95°C.

Luteinizing hormone receptor (LHr) expression was determined by quantitative, real-time PCR using the comparative cycle time method. This method measured the fold-change in mRNA for LHr between samples normalized to a reference gene. The reference gene used in the present

study was glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which has previously been used for mRNA semi-quantitation in similar protocols [6.30, 6.31]. Equine specific primers were generated for LHr (Thermo Fisher Scientific, Waltham, MA; Table 6.1) and GAPDH (Integrated DNA Technologies, Coralville, IA; Table 6.1). Primers for each gene were validated on equine luteal tissue, testes, and ovarian follicle cell aspirates. The PCR products were separated on 1% agarose gel and visualized with ethidium bromide.

Table 6.1 Genes of interest with primer sequences, accession numbers and amplicon length				
Genes of Interest	Forward primer 5' – 3'	Reverse primer 5' – 3'	GenBank Accession No.	Amplicon length
Luteinizing hormone receptor (LHr)	acgacactgattccctggag	acagcagtggcttgggtaag	XM_005599992.2	252 bp
GAPDH	caggtgtctcctgcgattt	cataaggtccaccaccctattg	NM_001163856.1	147 bp

Quantitative, real-time PCR was performed using commercially available reagents (SsoFast EvaGreen Supermix, Bio-Rad Laboratories, Hercules, CA) and analyzed on the CFX Connect Real-Time System (BioRad, Hercules, CA). A positive control (calibrator) was developed by combining mRNA from all control tissues and ovarian cell aspirates and transforming to cDNA as previously described. When appropriate, calibrator cDNA was “spiked” with purified PCR product (Monarch PCR & DNA Cleanup Kit, New England Biolabs, Ipswich, MA) such that a final concentration of 2 pg/μL was achieved for target genes. Samples were run in triplicate and consisted of 10 μL supermix, 1 μL each of forward and reverse primers, 4 μL water, and 4 μL sample cDNA, calibrator cDNA, or water as negative control. PCR amplifications consisted of 40 cycles of enzyme activation (95°C, 30 seconds), denaturation

(95°C, 5 seconds), annealing (53°C, 5 seconds for GAPDH, 57°C, 5 seconds for LHR) and extension (65°C, 5 sec). Melt curve analysis was performed on each sample to confirm specificity of amplification. The PCR products were separated on 1% agarose gel and visualized with ethidium bromide.

### **6.3.6 Data Analyses**

Data for dependent variables collected over many different time points (plasma concentrations of prolactin, LH, etc.) were analyzed by 2x2 factorial one-way analysis of variance (ANOVA) with repeated sampling using the general linear model of SAS (SAS Instit., Cary, NC). Relative gene expression was calculated using  $2^{-\Delta\Delta CT}$  [6.32]. Quantitative cycle values were normalized against the reference value of the housekeeping gene (GAPDH) within the same sample. These normalized values as well as follicular fluid factors and date to first ovulation were then analyzed by 2x2 factorial one-way ANOVA. Effects of follicle ablation and treatment were tested with animal within the interaction between follicle ablation and treatment as the error term. When appropriate, differences between treatment groups within time periods were tested for significance by the least significant difference test [6.33].

## **6.4 Results**

An ovarian response was defined as the occurrence of a pre-ovulatory follicle at least 35 mm in diameter within 28 days after the first sulpiride injection, which was then aspirated via ultrasound guided transvaginal aspiration. Complete follicle ablation had no effect ( $P > .1$ ) on many of the factors presented herein; therefore, when no effect was observed, means are presented simply as treatment versus control. When complete follicle ablation did have an effect ( $P < .05$ ), means are presented for all four groups comprising the 2x2 factorial. Of the 8 mares treated with ECP-sulpiride, 5 responded with early ovulatory sized follicles. Mean date to first

35 mm follicle was advanced ( $P = .03$ ) in mares treated with ECP-sulpiride ( $61 \pm 10$  days) compared to mares treated with ECP and vehicle only ( $96 \pm 10$  days). These days represent day of the calendar year. There was an interaction ( $P < .0001$ ) between treatment and day for mean plasma prolactin concentrations in samples obtained every 4 days (Figure 6.1). Prolactin was stimulated in sulpiride-treated mares for at least 12 days post treatment. No increase in prolactin was observed in mares treated with ECP and vegetable shortening. No differences ( $P > .1$ ) in the prolactin response to sulpiride were found between mares that responded with an early

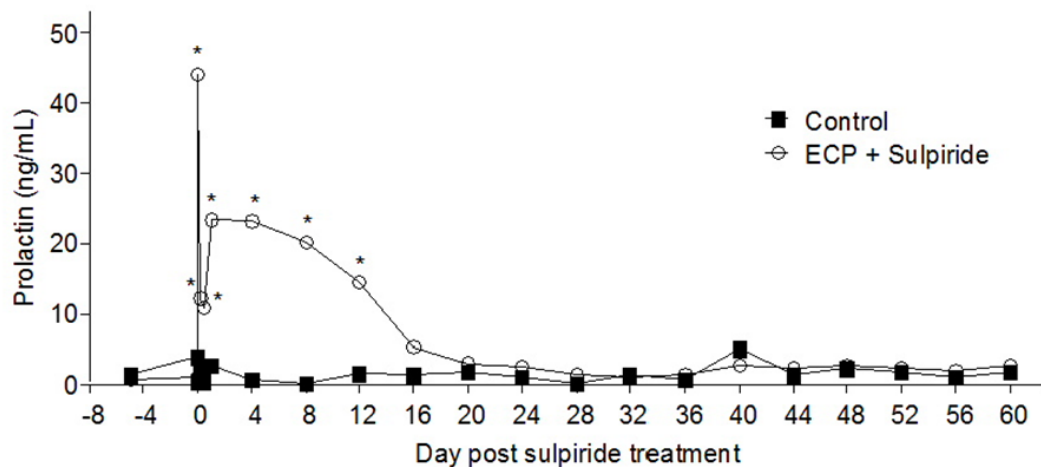


Figure 6.1 Mean concentrations of prolactin in samples obtained every four days in mares treated with 50 mg ECP and 3 g sulpiride in vegetable shortening (ECP + Sulpiride) or mares treated with 50 mg ECP and vegetable shortening only (Control). Sulpiride or vehicle was administered on day 0 and on day 7. Asterisks indicated differences ( $P < .0001$ ) between means at specified time points. Pooled standard error was 3.5 ng/mL.

ovulatory sized follicle (35 mm) and mares that did not. Complete follicle ablation prior to treatment with ECP-sulpiride had no effect ( $P > .1$ ) on the prolactin response.

Estradiol cypionate stimulated plasma LH in all mares ( $P < .05$ ; Figure 6.2A), with concentrations being higher in ECP-sulpiride treated mares on day 12. Ablation had no effect ( $P$



> .1) on LH response to ECP. Plasma LH was higher ( $P < .05$ ; Figure 6.2B) in treated mares that responded compared to mares that did not respond on days 8 and 12 post sulpiride treatment. Neither treatment nor ablation had an effect ( $P > .1$ ) on circulating FSH in samples collected every four days after treatment.

Concentrations of prolactin in follicular fluid aspirated from the first 35 mm follicle were greater ( $P = .05$ ) in mares treated with ECP-sulpiride compared to mares that received ECP and vehicle only. There was also an interaction ( $P = .06$ , Figure 6.3) between treatment and ablation

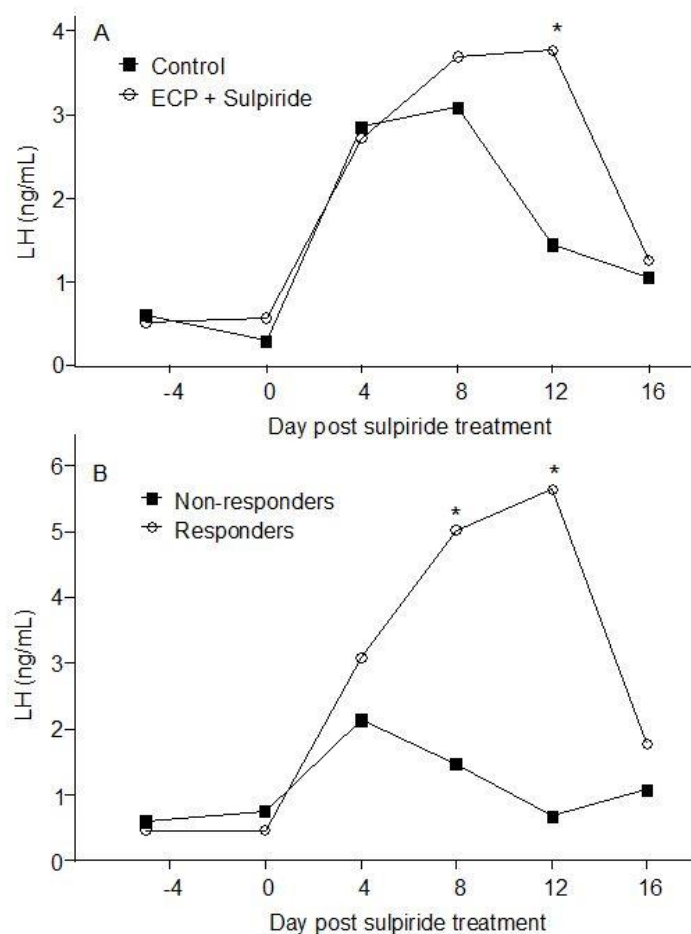


Figure 6.2. Mean plasma concentrations of LH in mares treated with 50 mg ECP and 3 g sulpiride in vegetable shortening (ECP + Sulpiride) or mares treated with 50 mg ECP and vegetable shortening only (Control; Panel A), and ECP-sulpiride treated mares who responded (Responders) with early ovulatory sized follicles compared to mares that did not (Non-responders; Panel B). Asterisks indicate differences between means at specified periods. Pooled SEM were 1.8 ng/mL for Panel A and 1.4 ng/mL for Panel B.

with concentrations being higher in mares treated with ECP-sulpiride that did not receive complete follicle ablation prior to treatment compared to treated mares that did. Follicular fluid concentrations of prolactin, estradiol, progesterone, leptin and IGF-1 are presented in Table 6.2.

Except for prolactin, no differences ( $P > .1$ ) were detected in the remaining hormones between mares treated with ECP-sulpiride compared to mares that received ECP and vehicle only. No effect of ablation ( $P > .1$ ) was observed for these hormones either.

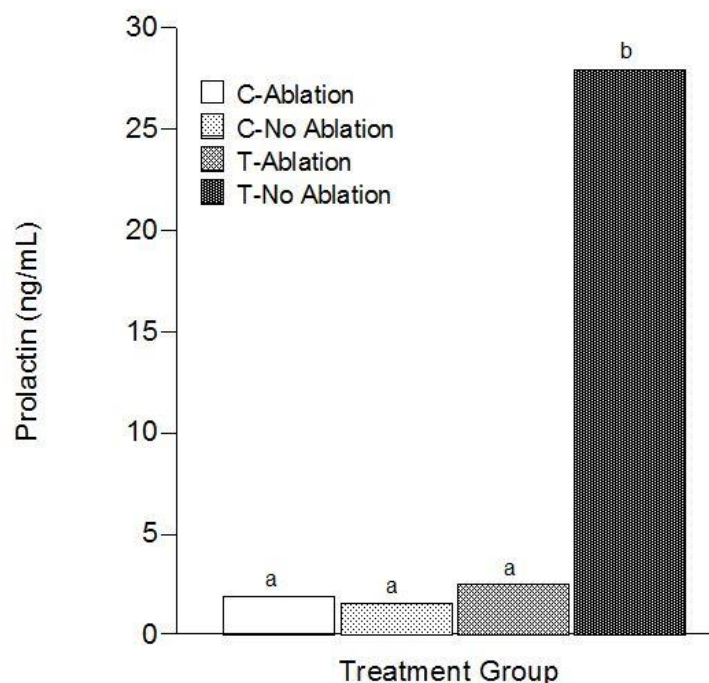


Figure 6.3 Mean concentrations of prolactin in follicular fluid aspirated from the first 35 mm follicle in mares treated with either ECP-sulpiride (T) or ECP and vehicle only (C) and received a complete follicle ablation prior to treatment (Ablation) or did not (No Ablation). Different letters indicate differences at  $P < .1$ . Pooled standard error of the mean was 7.3 ng/mL.

In plasma samples collected daily after aspiration, both LH and progesterone were higher ( $P < .01$ , Figure 6.4) in mares that were treated with ECP-sulpiride and were not ablated compared to ECP-only treated mares (both ablated and not) and to ECP-sulpiride treated mares that were ablated. Plasma estradiol and FSH were similar ( $P > .1$ ) in all aspirations.

Table 6.2 Mean concentrations of prolactin, estradiol, progesterone, leptin and IGF-1 in follicular fluid aspirated from the first 35 mm follicle.

	Mean $\pm$ SEM follicular fluid concentration (ng/mL)		Treatment effect
	Control	ECP - Sulpiride	P – value
Prolactin	1.8 $\pm$ 4.5 <sup>a</sup>	15.2 $\pm$ 3.7 <sup>b</sup>	.05
Estradiol	742.2 $\pm$ 217.5 <sup>a</sup>	861.9 $\pm$ 177.6 <sup>a</sup>	.63
Progesterone	55.7 $\pm$ 30.9 <sup>a</sup>	121.3 $\pm$ 25.2 <sup>a</sup>	.13
Leptin	1.05 $\pm$ 0.8 <sup>a</sup>	0.94 $\pm$ 0.7 <sup>a</sup>	.91
IGF-1	166.6 $\pm$ 30.7 <sup>a</sup>	180 $\pm$ 33.7 <sup>a</sup>	.76

Control = 50 mg ECP followed by treatment with vegetable shortening;

ECP - Sulpiride = 50 mg ECP followed by treatment with 3 g sulpiride in vegetable shortening. <sup>a-b</sup> Means within rows with no like superscript differ (P < .05).

Relative gene expression for LH receptors in cell aspirates from the first 35 mm or greater follicle was similar (P > .1, Figure 6.5) between all mares (treated versus control and ablation versus no ablation). Means for all four groups are shown to illustrate the consistency among groups.

## 6.5 Discussion

To date, this is the first study to assess changes in ovarian follicular factors in response to induced follicle growth after stimulation via prolactin elevation in winter. Ovarian success was defined by ovulation within 28 days post sulpiride treatment. Success rates of 50 to 89% have been achieved in previous experiments [6.6-6.10], depending upon the specific protocol used. The 89% success rate of Kelley et al. [6.6] was based on every-other-day injections of estradiol benzoate followed by daily sulpiride injections started 11 days later. A single injection of ECP followed by three treatments with sulpiride 5 days apart resulted in a success rate of 77% [6.10]. A success rate of 39% was reported by Oberhaus et al. [6.11] with only a single treatment of sulpiride either 1 or 5 days after ECP administration. In the current experiment, two sulpiride injections, administered 7 days apart, produced a success rate of 63% (5/8).

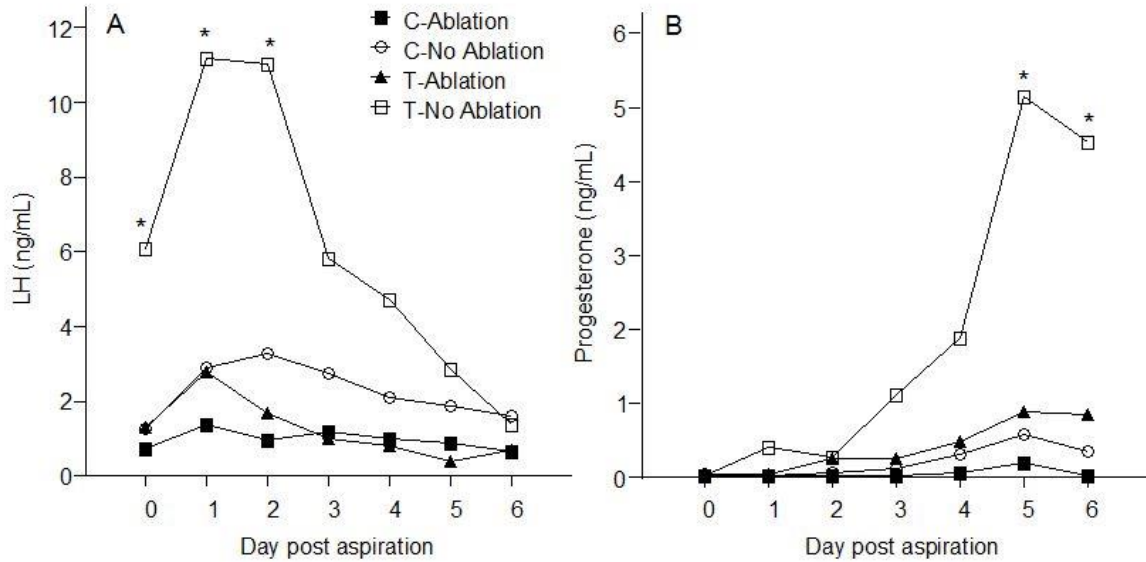


Figure 6.4 Mean plasma concentrations of LH (Panel A) and progesterone (Panel B) after aspiration of the first 35 mm follicle in mares treated with either ECP-sulpiride (T) or ECP and vehicle only (C) and received a complete follicle ablation prior to treatment (Ablation) or did not (No Ablation). Asterisks indicate differences ( $P < .01$ ) between specific time periods within each panel. Pooled standard errors of the means were 1 ng/mL for LH and 0.34 ng/mL for progesterone.

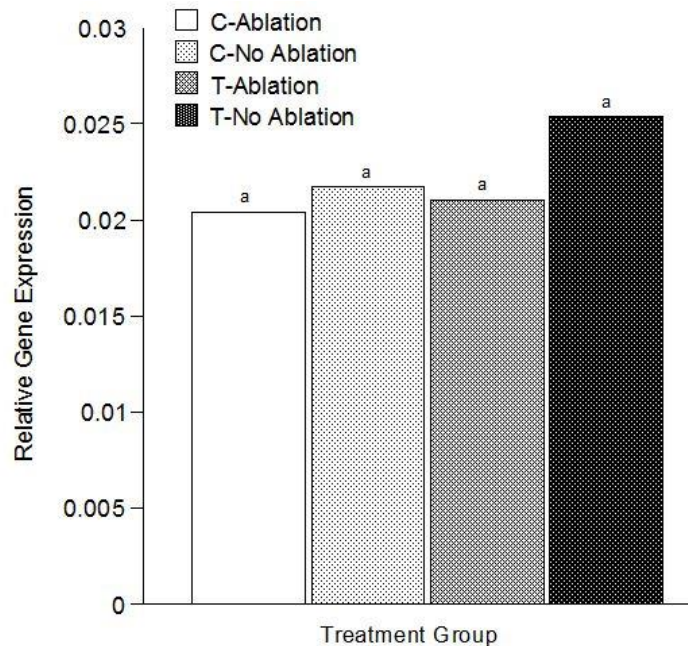


Figure 6.5 Relative gene expression for LH receptors after aspiration of the first 35 mm follicle in mares treated with either ECP-sulpiride (T) or ECP and vehicle only (C) and received a complete follicle ablation prior to treatment (Ablation) or did not (No Ablation). Like letters indicate no differences ( $P > .1$ ).

Timing of sulpiride treatment relative to ECP treatment has been investigated in several studies. Mitcham et al. [6.9] observed a tendency for prolactin response to be higher in mares receiving domperidone 1 day after ECP versus 6 days after ECP, whereas Oberhaus et al. [6.11] did not observe any differences in the prolactin response to sulpiride or the ovarian response between mares receiving sulpiride 1 versus 5 days after ECP. To simplify treatment, all treated mares in the present study received the first sulpiride injection 5 days after ECP. Timing of the first sulpiride treatment relative to ECP treatment does not appear to affect the ovarian response; however, a greater number of sulpiride treatments do appear to increase chances of a response.

Prolactin was stimulated in all sulpiride-treated mares for approximately 12 days by the injection of 3 g of sulpiride in vegetable shortening. The response to sulpiride did not differ between mares that developed 35 mm follicles early and mares that did not; moreover, treatment with ECP alone did not stimulate prolactin and resulted in no ovarian response. A second treatment of sulpiride 7 days after the first did not further increase prolactin in treated mares, although it appeared to sustain elevated prolactin for a longer period. This is compared to the 7-day stimulation of prolactin with one injection of sulpiride in a similar manner observed by Oberhaus et al. [6.11]. Given higher success rates in the present study with two sulpiride treatments versus one [6.11], it is likely that duration of prolactin stimulation, not magnitude, is contributory to ovarian response, as long as the prolactin response is above a given threshold. That is, a poor prolactin response is known to lead to no ovarian response.

All mares in this experiment were treated with 50 mg ECP; therefore, an increase in LH was observed in sulpiride-treated mares as well as mares treated with vehicle. Given the limited availability of seasonally anovulatory mares, it was decided that mares serving as controls would receive ECP as well to separate out any compounding effects of the ECP-sulpiride combination.

In past experiments [6.6, 6.10, 6.11], an increase in LH has not been observed in ECP or estradiol benzoate-treated mares until after sulpiride treatment, which led to the speculation that ECP and sulpiride, in combination, stimulate LH. In the present study, the LH response was virtually identical in sulpiride- and vehicle-treated mares and only differed 12 days after sulpiride treatment, which coincided with the first pre-ovulatory follicles that developed in 4 of the responding mares. Concentrations of LH were elevated in sulpiride-treated mares responding with a pre-ovulatory follicle compared to mares that did not 8 and 12 days after treatment with sulpiride. Again, this coincided with late stage follicular growth in responding mares; therefore, it is possible that LH is being stimulated by endogenous follicular estradiol as well as the exogenous estradiol via ECP injection.

Concentrations of prolactin, estradiol, progesterone, leptin, and IGF-1 were measured in follicular fluid collected from the first pre-ovulatory follicle in each mare. Prolactin was higher in sulpiride-treated mares that were not ablated prior to treatment compared to sulpiride-treated mares that were ablated and vehicle-treated mares both ablated and not. Two of the 5 responding mares had extremely high ( $> 30$  ng/mL) prolactin concentrations, which accounted for the statistical significance. When those mares were removed from the analysis (for comparison), the effects of treatment and ablation were no longer significant but there was still a tendency for sulpiride-treated mares to have higher prolactin concentrations in follicular fluid.

Concentrations of prolactin in follicular fluid have been shown to increase with follicle size in the mare and the rise in follicular fluid prolactin coincided with a surge in plasma prolactin observed during the periovulatory period [6.34]. Although it is unclear if the rise in plasma prolactin contributes to follicular fluid prolactin, it is likely that a robust prolactin response to sulpiride is stimulatory to ovarian follicle prolactin production.

Estradiol and progesterone concentrations within the follicles were variable among mares, but did not differ between mares that were treated with sulpiride and mares that received vehicle only. Low estradiol and progesterone concentrations have been observed in follicular fluid of seasonally transitional follicles [6.35], and it is current dogma that a deficiency in steroidogenesis by the early transitional follicle is responsible for the prolonged presence of an ovulatory-sized follicle that fails to ovulate [6.36]. Given that no difference was observed in follicular fluid estradiol and progesterone between treated and control mares, it appears that early-induced follicles are equally as competent in terms of steroid production as those occurring naturally in the spring.

Concentration of IGF-1 was measured in fluid collected from the first pre-ovulatory follicle due to its proposed role in initiating selection of the future dominant follicle [6.37, 6.38] and association with steroidogenesis [6.39]. Leptin was measured due to its negative relationship with reproduction in mares [6.40]. Both IGF-1 and leptin concentrations were similar in follicular fluid collected from the first pre-ovulatory follicle of sulpiride and vehicle-treated mares.

In daily plasma samples collected for 6 days after follicular aspiration, both LH and progesterone concentrations were higher in responding mares that were treated with sulpiride and not ablated prior to treatment than responding, sulpiride-treated mares that were ablated. Non-responding mares were excluded from the analysis given that they developed pre-ovulatory follicles outside the 28 day response period when prolactin was no longer stimulated. The goal of the analysis was to characterize LH and progesterone immediately after aspiration of early-induced follicles compared to those occurring naturally. The significant rise in LH can be attributed to one mare who had LH concentrations > 15 ng/mL. Excluding her from the analysis

revealed a similar rise in LH immediately after aspiration, much like that reported by Hinrichs et al. [6.41] and observed naturally after ovulation.

Plasma samples were collected daily after aspiration to determine if complete aspiration of a pre-ovulatory follicle would lead to luteinization of the follicle and, subsequently, progesterone production as has been previously reported in the mare [6.41, 6.42]. There was a post-aspiration increase in plasma progesterone, similar to a rise in early diestrus, in mares that responded and were not ablated prior to sulpiride treatment. Both mares in this group had a similar rise in progesterone. Non-responding, sulpiride-treated mares that were ablated, as well as vehicle-treated mares, both ablated and not, failed to produce progesterone concentrations > 1 ng/mL. For the 3 responding mares that were subjected to complete follicle ablation prior to treatment, ablation took place 17 days (2 mares) and 23 days (1 mare) prior to aspiration of the first pre-ovulatory follicle. Given the small number of responding mares in each group, these results are tenuous; however, the question arises as to what effect follicle ablation prior to treatment may have on the future of the next ovulatory follicle in terms of progesterone production.

For those vehicle-treated mares assigned to complete follicle ablation, ablation took place 60 days prior to aspiration of the first pre-ovulatory follicle at the earliest. Similar concentrations of follicular fluid progesterone in all groups of mares indicate similar steroidogenic capabilities; however, both vehicle-treated groups of mares (ablated vs. not ablated) failed to produce progesterone after aspiration of the first pre-ovulatory follicle. Since the pre-ovulatory follicle was aspirated, it is not known if that follicle was going to ovulate and form a fully functional CL. It is possible that the first naturally occurring, pre-ovulatory sized



follicle would have been anovulatory, and thereby not equipped for complete formation of a functional CL leading to insufficient plasma progesterone.

The most important factor contributing to a mare returning to cyclicity seems to involve 1) the re-establishment of LH secretion and its associated receptor [6.43] and 2) adequate plasma prolactin concentrations that may affect the LH receptor content. The first ovulatory-sized follicle of the season often grows to a diameter of > 40 mm before either regressing or eventually ovulating several days, or sometimes weeks, later (EL Oberhaus, personal observations). This led to the hypothesis that there is a failure of LH to induce ovulation either through insufficient pituitary production (perhaps by absence of estradiol feedback) or through inability of LH to exert its action on the ovarian follicle due to insufficient receptors. Given the stimulatory effects of prolactin on LH receptors in other species [6.18-6.22], the hypothesis that sulpiride upregulates LH receptors within the equine ovarian follicle was tested in this experiment. Luteinizing hormone receptor content, as determined by Q-PCR, was similar in early sulpiride-induced follicles and in those naturally occurring later in the season in control mares. This likely indicates that the early-induced, pre-ovulatory follicle can respond to circulating LH equally to follicles occurring naturally but later in the spring. One of the limitations of this experiment was the experimental paradigm only allowed for comparison of samples from ovarian follicles of like sizes in treated and control animals, which were unlikely to be collected during the same time period. In other words, a 35-mm follicle from a treated mare obtained in February would not normally provide a logical comparison to a 35-mm follicle from a control mare obtained in April. However, the data presented herein indicate that the treatment-induced, early follicles were indeed equivalent to their later, naturally occurring counterparts. Thus, prolactin may in fact be needed for the early-induced follicles to become LH responsive, and without it they would be

unresponsive and would not grow to pre-ovulatory size (as seen in mares that do not have a significant prolactin rise). What was not available for comparison was 35-mm or larger follicles from untreated mares at the same time.

In conclusion, combined ECP-sulpiride treatment stimulated circulating prolactin and hastened the date to first pre-ovulatory follicle, but degree of prolactin response was not a predictor of ovarian response, likely because it was beyond adequate in all treated mares. Sulpiride-treated mares that were not ablated had elevated plasma progesterone and LH after aspiration. There was a tendency for an increase in follicular fluid prolactin in sulpiride-treated mares. Ablation may inhibit maturity of the first pre-ovulatory follicle based on decreased progesterone and LH production, but does not inhibit growth and development of the first pre-ovulatory follicle. Most importantly, early-induced follicles appear to be equally mature (competent) to those occurring naturally in the spring in terms of steroid production and LH receptor mRNA. Because of this, ovulations from these induced follicles have in fact been shown to be fertile to a degree similar to those occurring naturally later in the spring [6.11].

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## OVERALL SUMMARY AND CONCLUSIONS

A series of experiments was performed to study the possible role for prolactin in the seasonal recrudescence of ovarian activity in the mare in winter. The first experiment (Chapter 3) was based on cabergoline administration to seasonally anovulatory mares to prevent the natural seasonal rise in endogenous plasma prolactin concentrations to assess whether reduced prolactin altered the onset of ovarian activity or the timing of the first ovulation. Although prolactin concentrations were altered in the early spring, basal prolactin concentrations apparently recovered from cabergoline suppression by the time of the next injection (10 days later), even though low-dose sulpiride stimulation of prolactin secretion was still suppressed by 85%. The subsequent experiment (Chapter 4) tested whether season affected the duration and degree of suppression produced by a standard dose of cabergoline. Mares were administered an injection of 1.5 mg/ 500 kg body weight of cabergoline in March, June, September, and December. Duration of suppression was not affected by month of assessment. Follow-up assessments with either low dose sulpiride (July) or TRH (October) challenges after administration of 5 mg cabergoline indicated that basal prolactin concentrations always rebounded faster (earlier) than secretagogue-induced secretion. The possibility of more than one lactotrope population with differing sensitivities to dopaminergic suppression is discussed.

The third experiment (Chapter 5) studied the possible physical and hormonal characteristics of seasonally anovulatory mares that might affect their response to a combined estradiol and sulpiride treatment for inducing ovarian activity and ovulation in winter. Mares from two farms were used. All mares received 50 mg of ECP followed by 3 g of sulpiride either 1 or 6 days later. Factors that were commonly associated with success (ovulation within 28 days) were adequate body condition, elevated plasma concentrations of leptin and insulin, and a greater

LH response to GnRH prior to the start of the experiment. Pregnancies were also produced in 75% of mares bred. It was concluded that, assuming a good prolactin response, factors associated with good nutrition seem to be most important to the positive ovarian response.

Finally, Experiment 4 (Chapter 6) studied the hormonal production and secretory aspects of the first pre-ovulatory (35 mm) follicle of the breeding season in seasonally anovulatory mares treated with either 50 mg ECP followed by 2 injections of 3 g sulpiride 5 and 12 days later, or 50 mg ECP followed by 2 injections of vehicle only 5 and 12 days later beginning in January. Date to first pre-ovulatory follicle was advanced in sulpiride-treated mares. Through assessment of follicular hormone production and LH receptor mRNA, it was concluded that early-induced follicles are equally as competent in terms of steroid production and ability to respond to circulating LH as pre-ovulatory follicles occurring naturally in spring.

These experiments support the hypothesis that an increase in circulating prolactin during seasonal anestrus can hasten follicular growth and advance the date to first ovulation in most mares. Failure to observe any perturbations on vernal transition by incomplete suppression of prolactin does not negate the decades of research which have detailed stimulatory effects of prolactin on follicular growth. And while a prolactin response to dopamine antagonists is necessary for early induced ovulation, magnitude of response is not an indicator of ovarian success (assuming the response is above some theoretical threshold). Other factors, such as nutrition and depth of anestrus, may mediate the ovarian response to the prolactin, whether it be naturally occurring or induced by estradiol and sulpiride.

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## **VITA**

Erin Lea Oberhaus, daughter of Cheryl Henson Oberhaus and Frank and Debra Lawson, was born in Carbondale, IL on November 23, 1983. Erin is the second youngest of 6 children: Kristan (Daniel) Oberhaus, Dustin (Amanda) Olson, Erik Oberhaus, Ericka (Scott) Dixon and Jarrod Oberhaus. Erin is aunt to 4 nieces, Emma, Caroline, Ava and Emily, and 3 nephews, Jacob, Chance and Zackary. Erin grew up in Williamsville, MO and later, Cape Girardeau, MO. She attended high school at Central High School in Cape Girardeau, MO where she graduated with honors in 2002. In 2007, Erin earned a Bachelor of Science degree in agribusiness from Southeast Missouri State University. She then pursued a Master of Science degree in animal science from Southern Illinois University-Carbondale, graduating in 2012. During the fall of 2012, Erin began working on her doctorate degree in animal and dairy sciences at Louisiana State University. She has accepted a position offer as assistant professor of animal sciences at Louisiana State University contingent upon her earning her degree.