INTERACTIVE ROLES OF GONADOTROPIN-RELEASING HORMONE AND RF-AMIDE RELATED PEPTIDE 3 IN ADENOHYPOPHYSEAL PHYSIOLOGY AND REPRODUCTION IN THE MARE

A Dissertation

by

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Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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May 2013

Major Subject: Physiology of Reproduction

ABSTRACT

The seasonal termination of ovarian cycles in mares initiated near the time of the autumnal equinox is a significant managerial issue for horse breeders world-wide. Studies presented herein had two over-arching aims. In Aim I, objectives were to develop the principals needed to apply gonadotropin-releasing hormone (GnRH) therapeutics for routinely establishing pregnancies in the winter anovulatory mare. We first tested the hypothesis that continuous administration of native GnRH, beginning in either early February or March, would induce ovulation without reversion to an anovulatory state following treatment withdrawal. Continuous 28-d treatments elevated circulating luteinizing hormone (LH) and stimulated spontaneous ovulation much earlier than controls. However, mares treated only in February ceased ovarian cycles at termination of treatment. In contrast, mares administered GnRH in March continued to exhibit estrous cycles. Thus, we concluded that GnRH treatment must continue through March to ensure continued escape from winter anovulation. We then tested the hypothesis that the Julian day of conception could be accelerated in winter anovulatory mares treated continuously with native GnRH for 56 d beginning on February 1. Indeed, GnRH treatment caused a marked increase in the frequency of pregnancy compared to controls. Data illustrated that continuous administration of native GnRH is a practical and highly efficient option for managing seasonal anovulation. In Aim II, we examined hypothalamic distribution, adenohyphyseal receptor gene expression, and ligand functionality of RFRP3 in the mare during the breeding and non-breeding seasons.

Hypothalamic RFRP3 mRNA was detected in the mare; however, neither hypothalamic expression of RFRP3 nor its anterior pituitary receptor differed between reproductive states. We then used equine adenohypophyseal cell culture to test the hypothesis that RFRP3 reduces the responsiveness of the equine gonadotrope to GnRH. Addition of RFRP3 to cell culture failed to counter the effects of GnRH. Finally, the effects of a RFRP3 receptor-signaling antagonist (RF9) were examined in winter anovulatory mares. A robust increase in circulating concentrations of LH relative to controls was observed in response to RF9 treatments, but treatments had no effect on adenohypophyseal responsiveness to GnRH. Data provide indirect evidence that antagonism of the RFRP3 system by RF9 may be at the GnRH neuronal level.

DEDICATIONS

To the mentors who molded me,

the peers that put up with me,

and my family for their tireless support.

NOMENCLATURE

3V……...……...Third ventricle

AI……...……...Artificial insemination

BCS……Body condition score

BSE……...…..Breeding soundness exam

CG……..……Chorionic gonadotropin

CL……...……...…Corpus luteum

DAG……...…….……..Diacylglycerol

DMH……..……...Dorsomedial nucleus of the hypothalamus

EPE……..……..…….Equine pituitary extract

FSH……...…….…….Follicle stimulating hormone

FX……...……...…….Fornix

GnIH……...……Gonadotropin-inhibitory hormone

GnRH……..……Gonadotropin-releasing hormone

GnRHa……..……..Gonadotropin-releasing hormone agonist

GPR147……...……...G-protein coupled receptor 147

HIOMT……..……...…….Hydroxy-indole-0-methyl transferase

ICS……...……Intercavernous sinus

IP3……...…….……Inositol triphosphate

LH……..…….Luteinizing hormone

MAPKs…….....Mitogen-activated protein kinases

MT……..Mammillothalamic tract

NMA…….…….N-methyl-DL-aspartic acid

OT……...……..……..Optic tract

POA……..……...Preoptic area

PLC……...…….….Phospholipase c

PKC……...……..……Protein kinase c

PVN……...……Paraventricular nucleus of the hypothalamus

RF9……...……..Adamantanecarbonyl-Arg-Phe-NH4

RFRP3……..……...Arginine phenylalanine related peptide 3

RIA……...…….…Radioimmunoassay

rpPRL……...……...Recombinant porcine prolactin

SAS……...……...….Statistical analysis software

SCN……...……Suprachiasmatic nucleus

SNAT……...……...…..Serotonin-N-acetyltransferase

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CHAPTER I

INTRODUCTION

Reproductive seasonality in the mare is characterized by an abrupt and marked decline in adenohypophyseal synthesis and secretion of luteinizing hormone (LH) near the autumnal equinox (Ginther, 1992). This results ultimately in the cessation of ovarian cycles in at least 85% of standard horse mares by the time of the winter solstice. Resumption of normal anterior pituitary and ovarian function follows approximately 3 to 4 mo later (Ginther, 1992). Length of the winter anovulatory season can be reduced by 1) provision of supplemental lighting beginning near the winter solstice to extend day-length to 15 to 16 h (Ginther, 1992), or 2) treatment with native gonadotropin-releasing hormone (GnRH) to stimulate synthesis and secretion of LH (Johnson, 1987; Becker and Johnson, 1992). Currently, the provision of supplemental lighting is the only practical option available to the equine industry.

Johnson (1987) first demonstrated that hourly intravenous injections of native GnRH to anovulatory mares in January, at doses as low as 2 μg/h, rapidly increase circulating concentrations of LH, stimulates follicular development, and causes spontaneous ovulation within 2 wk. Similar but delayed responses were attained using continuous subcutaneous infusions (Hyland and Jeffcott, 1988; Becker and Johnson, 1992); however, 10 to 30-fold greater doses of GnRH were required to elicit a LH response. Importantly, and in contrast to effects in other species, continuous treatment of mares with native GnRH at dose rates as great as 250 μg/h does not result in pituitary

desensitization (Porter et al., 1997). Therefore, continuous, high-dose infusions of native hormone remain functionally stimulatory in the horse. These relationships do not hold true for synthetic, long-acting GnRH agonists (Fitzgerald et al., 1993; Mumford et al., 1994) which, when given continuously, result in pituitary desensitization. Thus, the decision by the industry to focus primarily on synthetic agonists for commercially-targeted applications, while optimizing proprietary potential, have failed as practical therapeutic agents in the transitional mare.

From a purely endocrine perspective, data support the hypothesis that reductions in hypothalamic GnRH release during the winter anovulatory period account for the characteristic reduction in synthesis (Hart et al., 1984) and release (Sharp & Grubaugh, 1987) of LH during this period. However, Cooper et al. (2006) measured GnRH secretion patterns in pituitary venous effluent of mares during 4 seasons using the intercavernous sinus (ICS) cannulation technique, yielding results that contradict existing dogma. Results indicated that ICS concentrations of GnRH, and the frequency and amplitude of GnRH pulses, were invariant across seasons. Thus, although contrary to accepted dogma, it is possible that the reduction in secretion of LH occurring about the time of the autumnal equinox may not be regulated solely through reduced endogenous secretion of GnRH. Velez et al. (2008) demonstrated that the anovulatory mare is less responsive to GnRH following the winter solstice than during the period just before or at the winter solstice. Thus, it was concluded that the length of exposure to a non-permissive photoperiod has a greater negative impact on adenohypophyseal responsiveness to GnRH than absolute day-length at the time of GnRH treatment.

However, it remains unclear as to whether this is a consequence of a longer period of deficits in GnRH stimulation or maximal effects of a negative modulator of gonadotrope function.

Mechanisms through which gonadotrope function could be impeded during winter anovulation have not been determined in the mare. However, it could include effects mediated through a recently-discovered neuropeptide family, arginine phenylalanine-amide related peptide (RFRP). Members of the RFRP family have been linked to reproductive seasonality in seasonal breeding birds and some mammalian species, with marked changes in RFRP gene expression and peptide accumulation in the hypothalamus with changing photoperiod (Tsutsui et al., 2000; Ubuka et al., 2005; Clarke et al., 2008; Revel et al., 2008). In birds, one RFRP sequence (RFRP3) has now been termed gonadotropin-inhibiting hormone (GnIH). The latter directly suppresses secretion of LH and adenohypophyseal responsiveness to GnRH in birds and several mammalian species (Tsutsui et al., 2000; Kriegsfeld el al., 2006). Therefore, it is possible that an equine homolog of this peptide could play a major role in negatively regulating reproductive seasonality in the mare.

This dissertation consists of two over-arching aims as it relates to the stimulatory (GnRH) and inhibitory (RFRP3) control of the reproductive neuroendocrine axis of the mare. The first over-arching aim consists of experiments designed to better understand the ability of continuously-administered, native GnRH to regulate adenohypophyseal function during the winter anovulatory period. This information will then be used to optimize practical procedures for the commercial use of GnRH to manage seasonal

anovulation in a North American management setting. In the second over-arching aim, we examined hypothalamic distribution, adenohyphyseal receptor gene expression, and ligand functionality of RFRP3 in the mare during the breeding and non-breeding seasons. These data will provide the groundwork for intervention strategies utilizing antagonists of the RFRP3 signaling cascade to disinhibit the effects of an inhibitory photoperiod in the winter anovulatory mare.

CHAPTER II

REVIEW OF LITERATURE

2.1 Neuroanatomical tracts underlying seasonality in the horse

The control of reproductive seasonality in mammals is ultimately regulated by the environmental cue of photoperiod. In mares, photoperiod is recognized by retinal photoreceptors and transmitted as neuronal signals along the optic nerve, decussate at the optic chiasm to reach the supra chiasmic nuclei (Sharp et al., 1984) and superior cervical ganglia (Sharp et al., 1979), and eventually modulate function of the pineal gland (Goldman, 2001). Pinealocytes intercept this neuronal signal as exocytosed norepinephrine that binds to cellular membrane-bound β-adrenergic receptors (Hadley & Levine, 2007). Upon receptor binding, intracellular mechanisms convert tryptophan to melatonin that can eventually be released into the surrounding capillary network or cerebrospinal fluid.

2.2 Melatonin synthesis

Mechanisms that regulate melatonin synthesis within the pinealocyte begin with the conversion of tryptophan to serotonin via enzymatic conversion by tryptophan hydroxylase and aromatic amino acid decarboxylase (Young and Anderson, 1982). Interestingly, tryptophan hydroxylase activity is increased during the nocturnal period (Shibuya et al., 1978; Sitaram and Lees, 1978) allowing for increased melatonin production. Serotonin is then enzymatically converted to N-acetylserotonin via

serotonin-N-acetyltransferase (SNAT). Similar to tryptophan hydroxylase, concentrations of SNAT are very low within the pineal gland during the day (Buda and Klein, 1978). Nocturnally, SNAT activity is increased by norepinephrine released from neuronal terminals that synapse at the pineal gland (Buda and Klein, 1978). Norepinephrine also activates the intracellular pinealocyte adenylate cyclase-cAMP second messenger system through stimulation of membrane β-adrenoreceptors (Auerbach et al., 1981). Finally, hydroxy-indole-0-methyl transferase (HIOMT) enzymatically converts N-acetylserotonin to melatonin (Buda and Klein, 1978). Importantly, pineal concentrations of HIOMT remain elevated throughout the day (Reiter, 1991). Therefore, this enzyme is not believed to be rate limiting in biosynthesis of melatonin (Reiter, 1991).

2.3 Melatonin control of reproductive seasonality

Circulating concentrations of melatonin are entrained in a circadian rhythm, with the greatest concentrations observed during dark periods (Sharp & Cleaver, 1993). Thus, seasonal breeding species appear to perceive environmental cues through changes in circulating melatonin that influence reproduction; however, much debate surrounds the issue of melatonin action. In the horse, Nonno et al (1995) reported no melatonin receptors within the suprachiasmatic nucleus (SCN) as noted in other long-day breeders (Siberian hamster; Weaver et al., 1991; Reppert et al., 1994), short-day breeders (ewe; Bittman and Weaver, 1990), and rat (Reppert et al., 1994). Instead, it was suggested that seasonal influence is regulated through melatonin binding to receptors located in the pars

tuberalis, pars distalis, and pars intermedia, as 2-[^{125}I] iodomelatonin binding is absent in the equine hypothalamus (Nonno et al., 1995). In contrast, Stankov et al (1991) reported low, but significant melatonin receptor content within the SCN, preoptic area (POA), and cortical regions. Further, these binding sites were of high affinity, low capacity and high specificity (Stankov et al., 1991). Localization of melatonin receptors within the SCN supports the finding that under constant exposure of light, ovariectomized pony mares have increased hypothalamic GnRH content and subsequently circulating concentrations of LH (Cleaver et al., 1991). However, while exogenous, subcutaneous administration of melatonin to ovariectomized pony mares during the breeding season reduced hypothalamic GnRH content (Strauss et al, 1979) it did not alter circulating LH concentrations in ovariectomized or intact pony mares (Peltier et al., 1997). The report of Strauss et al. (1979) can be interpreted to indicate that GnRH synthesis is reduced due to the effects of melatonin which may result in a similar decline in GnRH release. However, this does not address the failure of exogenous melatonin to suppress circulating concentrations of LH (Peltier et al., 1997) which would be interpreted to indicate that release of GnRH is not affected by melatonin. Instead, it suggests that an inhibitory modulator may be affecting gonadotropin synthesis and/or release which is in contrast to dogma indicating that a deficiency in GnRH synthesis and/or secretion is the central factor driving the demise of seasonal reproductive cyclicity (Hart et al., 1984; Grubaugh et al., 1982; Barrell et al., 1992; Irvine et al., 2000).

2.4 GnRH control of reproductive seasonality

Secreted GnRH travels from the hypothalamus through the hypothalamo-hypophyseal portal system targeting its G-protein coupled receptor on gonadotropes (Irvine & Alexander, 1993). Gonadotropin-releasing hormone receptor number is invariant across seasons in the mare (Hart et al., 1984). Upon GnRH binding to its receptor, the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), are synthesized and secreted to induce ovarian follicular growth and ovulation. This mechanistic regulation of gonadotropes allows for the potential of therapeutic application of GnRH to modify reproductive cyclicity, particularly in the mare which is resistant to adenohypophyseal refractoriness upon continuous infusion of native GnRH (see below).

2.5 Industry-induced problem

With the establishment of the "official" birth date set as January 1 by the English Jockey Club in 1833 and subsequently adopted worldwide by the majority of modern breed associations, performance incentives are associated with earlier foaling because earlier foals are more mature during their early years of competition (Ginther, 1992; Langlois and Blouin, 1996). However, mares are seasonally polyestrous, with a period of reproductive competence occurring from approximately April to October (Northern Hemisphere). Thus, manipulation of the reproductive axis is required for foals to be born early in the calendar year (Ginther, 1992). Currently, only two options for hastening reproductive cyclicity exist in the winter anovulatory mare.

2.6 Methods to induce ovulation precociously

2.6.1 Extend photoperiod

The first, and most widely used option to hasten reproductive cyclicity is the supplementation of lighting beginning around the time of the winter solstice (December 21) to artificially extend day-length to 15 to 16 h (Ginther, 1992). This methodology was first described by Burkhardt (1947) as an effective, noninvasive means to advance the breeding season. However, it is important to note that this treatment does not shorten, but merely advances the transition period (McCue et al., 2007) with interval from initiation of light supplementation to ovulation ranging from approximately 60 to 90 d (Ginther, 1992). Further, as light treatments require the interruption in endogenous, nocturnal melatonin secretion, this treatment must be 1) applied at dusk, reducing the period of darkness to 8 to 9 h (Burkhardt, 1947) or 2) applied for 1 to 2 h during the nocturnal photosensitive phase that begins from 8 to 9.5 h after the onset of darkness (Palmer et al. 1982; Malinowski et al. 1985; Gulliaume et al. 2000). Also, care must be taken when removing mares from supplemental lighting when ambient photoperiod is short, as mares may return to a winter anovulatory state (Scoggin et al., 2000) following induced ovulation.

The second option to hasten reproductive cyclicity is the application of pharmacological treatments to stimulate the synthesis and secretion of LH by gonadotropes, increase circulating concentrations of exogenous gonadotropins or gonadotropin-like compounds, or enhance the ability of the ovary to respond to limited gonadotropic stimuli.

2.6.2 Prolactin

Circulating concentrations of prolactin are inversely related to concentrations of melatonin, with the greatest concentrations of prolactin observed during the summer (Johnson, 1986; Thompson et al., 1986). In fact, recombinant porcine prolactin (rpPRL) induced rapid increases in circulating prolactin and follicular growth, while reducing mean interval to induced ovulation (21 d) in winter anovulatory mares (Thompson et al., 1997). However, it has been proposed that large, exogenous doses of prolactin are acting at the level of the ovary to induce rapid follicular growth and ovulation (Nequin et al., 1993; Thompson et al., 1997) and not on enhancing gonadotrope function. Further, Thompson et al (1997) reported that all rpPRL treated mares exhibited antibodies to the treatment by the 28th day of rpPRL injection. However, these studies were performed using a limited number of animals and recombinant equine prolactin treatments have not been pursued commercially.

2.6.3 Dopamine antagonists

As a means to circumvent antibody production, treatments that enhance endogenous prolactin secretion have been developed. One such method involves the administration of dopamine antagonists such as sulpiride and domperidone (Olin et al., 1991). These compounds antagonize the suppressive effects of dopamine during the winter anovulatory period and allow for circulating concentrations of prolactin to increase (Cross et al., 1995). Unlike reports using rpPRL, results utilizing dopamine antagonists to enhance endogenous prolactin secretion are conflicting. Some

experiments reported only follicular growth (Nequin et al., 1993), whereas others have reported follicular development, shortened interval to ovulation, normal progesterone profiles, and fertile ovulations with large, daily doses (sulpiride, 200 mg/mare to 1 mg/kg BW; Besognet et al., 1996; Besognet et al., 1997). However, all sulpiride treated mares that did not become pregnant, returned to winter anovulation. In contrast to the above observations, the same treatment (1 mg/kg BW) administered to mares in deep winter anovulation failed to stimulate follicle development or ovulation in spite of elevating circulating concentrations of prolactin (Donadeu and Thompson, 2002). Importantly, mares in this study were not maintained under artificial photoperiod as were the aforementioned studies. Interestingly, domperidone at a similar dose (1.1 mg/kg BW) was able to induce follicular growth, reduce the interval to ovulation, result in normal progesterone profiles, and elevate prolactin in deep winter anovulatory mares under ambient photoperiod (Brendemuehl and Cross, 1996; Brendemuehl and Cross, 2000). Further, this treatment had no effect on FSH or LH secretion, yet 75% of treated mares continued to cycle following treatment withdrawal. It was hypothesized that dopamine antagonists allow for increased circulating concentrations of prolactin which may increase ovarian gonadotropin receptors, thus increasing responsiveness to an existing environment of low circulating concentrations of endogenous gonadotropins (Duchamp and Daels, 2002). In spite of the potential advantages of using dopamine antagonists to reverse seasonal anovulation in the mare, responsiveness to treatment has been inconsistent and seems to be dependent on depth of anovulation and the concomitant extension of photoperiod. The roles of other catecholamines, such as

norepinephrine and epinephrine, have not been thoroughly investigated in the anovulatory mare. However, these compounds have been indirectly implicated in reducing the secretion of GnRH during the non-breeding season using an α-adrenergic agonist (Xylazine) to increase pulse frequency of FSH and LH (Fitzgerald and Mellbye, 1988). Therefore, catecholamine antagonists may serve some function in reversing seasonal anovulation.

2.6.4 Endogenous opioid peptides

Increased opioidergic inhibitory tone occurs during the non-breeding season and mares that ovulate spontaneously during the non-breeding season exhibit reduced opioid inhibition (Turner et al., 1995; Davison et al., 1998). In fact, blockade of this system through administration of the opioid antagonist naloxone increases the secretion of LH in the seasonally anovulatory mare (Aurich et al., 1994). Sharp et al. (1985) was not able to suppress secretion of LH using a large dose (2 mg/kg BW) of naloxone; however, this is most likely a function of the bell-shaped response curve of naloxone (Irvine et al., 1994). Therefore, it is believed that opioids inhibit the hypothalamic pituitary axis of the mare during times of reduced photoperiod and this inhibition is reduced as the winter anovulatory mare transitions into the breeding season (Turner et al., 1995; Davison et al., 1998). It has been demonstrated in other species that opioid-induced inhibition of LH secretion is regulated by inhibiting the secretion of hypothalamic GnRH (Cicero et al., 1979; Kalra, 1981; Wilkes and Yen, 1981; Grossman et al., 1981) but this has not been confirmed in the mare.

2.6.5 Neuro-excitatory amino acids

It has been proposed that insufficient GnRH neuronal stimulation drives the seasonal reduction in gonadal activity. To test this, Fitzgerald (1996) treated intact and ovariectomized mares during the non-breeding season with *N*-methyl-DL-aspartic acid (NMA) to stimulate neuro-excitatory amino acid receptors. This compound was able to stimulate secretion of gonadotropins in intact and ovariectomized mares. However, Fitzgerald and Davison (1997) concluded that neuro-excitatory amino acids do not play a role in seasonal regulation of reproduction as NMA resulted in a similar level of gonadotropin stimulation in ovulatory and anovulatory mares during the non-breeding season. Further, the large dose (5 mg/kg BW) of NMA required to induce sufficient gonadotropin secretion results in excessive excitability that put both mare and personnel in danger (Fitzgerald, 1996).

2.6.6 Gonadotropins and gonadotropin-like compounds

Use of equine pituitary extract (EPE) has proven highly effective (87 to 100%) in hastening follicular growth and ovulation induction in deep winter anovulatory mares (Douglas et al., 1974; Lapin and Ginther, 1977). However, Woods and Ginther (1982) demonstrated that treatment with EPE is more effective in late transitional mares as the interval from treatment onset to ovulation is reduced in mares with greater initial follicle size. Further two important factors need to be taken into consideration: 1) treatment with EPE requires more intense management as EPE results in multiple ovulations in 44 to 58% of treated mares (Douglas et al., 1974; Woods and Ginther, 1982) and 2) 55% of

mares treated with EPE that failed to become pregnant returned to winter anovulation following treatment withdrawal (Woods and Ginther, 1982). In addition, EPE is not used in practice as a commercial source is not available and collection of equine pituitaries is difficult since U.S. equine slaughter facilities have been closed. Recently, recombinant equine gonadotropins (reFSH and reLH) and recombinant equine gonadotropin-like (recombinant equine chorionic gonadotropin; reCG) treatments have demonstrated promise for inducing follicular growth (reFSH; Jennings et al., 2009) and ovulation (reLH and reCG; Niswender et al., 2006; Yoon et al., 2007). However, published studies have not determined directly the ability of such treatments to induce follicular development and ovulation in the winter anovulatory mare. Meyers-Brown (2010) has demonstrated that concomitant treatment with reFSH and reLH shortens the interval to ovulation in transitional mares; however, a limited number of mares were utilized in this study and multiple ovulations were observed.

2.6.7 Progesterone and progesterone combinations

Conflicting evidence exists as to the efficacy of advancing first ovulation using natural and synthetic progesterone treatments in the winter anovulatory mare. It is known that progesterone treatments suppress the irregular periods of estrous behavior characteristic of the transitional mare (Turner et al., 1981) and advance the synchronous interval to first ovulation in transitional mares (Squires et al., 1979; Allen et al., 1980; Squires, 1993; Nagy et al., 1998a,b). However, progesterone treatments will not advance ovulation, consistently in the deep seasonally anovulatory or early transitional mare

(Squires et al., 1979; Allen et al. 1980; Turner et al., 1981; Alexander and Irvine, 1991). Further, the combined treatment of progesterone and estradiol 17-β is not more effective than progesterone alone (Wiepz et al., 1988). Therefore, as progesterone treatments are not consistently effective for initiating ovarian cyclicity in a majority of mares, the widespread acceptance of progesterone treatments have not been observed.

2.6.8 Gonadotropin-releasing hormone

It was first demonstrated by Johnson (1987) that intravenous administration of native GnRH in hourly pulses to anovulatory mares in January would result in increased circulating concentrations of LH, development of a preovulatory follicle, and in most cases spontaneous ovulation within 2 wk. This occurred at doses as low as 2 μg/h, when administered in hourly pulses. Similar responses could be attained using the continuous, subcutaneous route, but doses 10 to 50-fold greater were required to be effective (Becker and Johnson, 1992). Therefore, while existing dogma indicates that continuously-applied GnRH treatment does not down-regulate the GnRH receptor in horses, its pulsatile administration clearly optimizes gonadotrope responsiveness. However, the application of intravenous, pulsatile treatments are not practical for routine use; therefore, subcutaneous administration is a suitable alternative that elicits the desired gonadal response. This does not hold true for synthetic, long-acting GnRH agonists (Fitzgerald et al., 1993; Mumford et al., 1994; Farquhar et al., 2001). Further, withdrawal of GnRH agonist treatment following induced ovulation results in embryonic loss (Bergfelt & Ginther, 1992). Universal attempts during the last 15 yr to utilize these potent agonists

(because of their potential proprietary value) may have undermined scientific progress and commercialization of an existing technology.

Only one serious attempt at using native GnRH in field trials was reported before our own laboratory embarked on the "rediscovery" of GnRH therapeutics in the anovulatory mare. The study was reported from New Zealand in the 1980's that demonstrated subcutaneous infusion of native GnRH via osmotic minipumps at a rate of 40 to 60 μg/h for 28 d during late spring transition (equivalent to April in North America) accelerated first ovulation by approximately 5 wk (Hyland & Jeffcott, 1988). Early work in our laboratory, using similar pumps, demonstrated that subcutaneous delivery of native GnRH at a very low rate (2.5 to 5 μg/h) can adequately stimulate secretion of LH to accelerate the onset of ovulatory cycles and breeding activity in persistently anovulatory mares after April 1 (Williams et al., 2007). However, chronic treatment of mares at these low doses, beginning in October and continuing through March, was unable to avert the development of seasonal anovulation, and did not accelerate spring transition (Collins et al., 2007).

Subsequently, it was questioned whether there were differences in anterior pituitary responsiveness to GnRH before and after the winter solstice (Velez et al., 2008). Velez et al. (2008) hypothesized that the adenohypophysis of the winter anovulatory mare is less responsive to GnRH stimulation at the time of the winter solstice (shortest day) compared to a period following two months of increasing photoperiod (mid- to late-February). Mares were treated subcutaneously with native GnRH using osmotic pumps at doses of 0, 20, or 100 μg/h. Results demonstrated that

anovulatory mares are actually less responsive to GnRH during the first 2 mo following the winter solstice than during the period just before or at the winter solstice. Mares responded to the 100 μg/h dose both before and after the winter solstice, but responded to the 20 μg/h dose only in December. Development of ovulatory follicles occurred during both periods in response to 100 μg/h, but follicle size did not change compared to controls in response to the 20-μg/h dose at either time period. Thus, it was concluded that the length of exposure to a non-permissive photoperiod has a greater negative impact on adenohypophyseal responsiveness to GnRH than absolute day-length (i.e., winter solstice) at the time of GnRH treatment. Most importantly, it appears that subcutaneous infusion of GnRH at a rate of 100 μg/h successfully induces synthesis and secretion of LH, resulting in development of preovulatory follicles (≥ 35 mm), and a greater proportion of spontaneous ovulations (7/10) at both times tested compared to controls and mares treated with 20 μg GnRH/h. However, two main managerial questions remain 1) what is the optimal time to begin treatment to hasten the spring reproductive transition to optimize subsequent foaling dates and 2) will mares treated with continuous native GnRH beginning prior to the spring reproductive transition maintain ovarian cyclicity and/or pregnancy following treatment removal?

2.7 Challenging dogma

If dogma is correct and GnRH is truly limiting in the winter anovulatory mare, then continuous administration of native GnRH (as opposed to agonists that are known to cause receptor down-regulation) might prevent the fall transition from an ovulatory to

anovulatory state in the mare. Using relatively small doses of native GnRH, Collins et al. (2007) continuously infused GnRH beginning before the fall transition (late September/early October) and failed to prevent winter anovulation or to hasten reproductive transition in the spring. Further, using the technique of intercavernous sinus (ICS) cannulation, Cooper et al. (2006) indicated that GnRH concentration, pulsatility, and amplitude do not vary across seasons in pituitary venous effluent illustrating that the reduction in secretion of LH occurring about the time of autumnal equinox may not be regulated through reduced secretion of GnRH. However, previous work involving the measurement of circulating concentration of GnRH indicates variation across seasons (Sharp & Grubaugh, 1987), while results pertaining to hypothalamic content (Strauss et al., 1979; Hart et al., 1984) are contradictory. Collectively, there has been an accrual of evidence indicating the possibility that decreased sensitivity of adenohypophyseal gonadotropes to GnRH may play a greater role in the development of winter anovulation than a decline in endogenous secretion of GnRH (Cooper et al., 2006; Collins et al., 2007; Velez et al., 2008). Regardless of the nuances contributing to this theoretical debate, there is one simple and incontrovertibly clear fact: native GnRH can reverse seasonal anovulation in the mare if applied at the appropriate dose and for an adequate duration (Hyland & Jeffcott, 1988; Becker and Johnson, 1992; Velez et al., 2008).

2.8 Inhibitory peptides as potential regulators of reproductive seasonality

If one is to challenge currently accepted dogma regarding neuroendocrine control of reproductive seasonality in the equine, a suitable alternative must be postulated. This

would require a molecule capable of altering the responsiveness of the gonadotropes to GnRH, resulting in the 6 to 10-fold decline in LH and onset of the anovulatory season. This molecule would act by inhibiting signals that result in GnRH-stimulated LH release. The mechanisms could involve modulation of GnRH receptor function or intracellular signaling pathways. The presence of such a molecule and the nature of its signaling remains to be elucidated in the equine but may include a recently-discovered neuropeptide family, arginine phenylalanine-amide related peptide (RFRP). This family of neuropeptides are homologs of the avian peptide, gonadotropin-inhibiting hormone (GnIH).

The first vertebrate in which GnIH was isolated was in Japanese quail and was aptly named for its ability to inhibit adenohypophyseal synthesis and secretion of LH in a dose-dependent manner *in vitro* and *in vivo* (Tsutsui et al., 2000; Ubuka et al., 2006). Both GnIH and RFRP have been linked to the regulation of reproductive seasonality in seasonal breeding species (rodents, avian, and ovine), with marked changes in GnIH/RFRP gene expression and peptide accumulation observed in the hypothalamus in response to changing photoperiod (Tsutsui et al., 2000; Ubuka et al., 2005; Clarke et al., 2008; Revel et al., 2008). Moreover, RFRP directly suppresses secretion of LH and hypophyseal responsiveness to GnRH in those species (Tsutsui et al., 2000; Kriegsfeld el al., 2006). This family of peptides functions through the GnIH receptor that belongs to the G protein-coupled receptor superfamily. In the avian, GnIH receptor mRNA is expressed in the hypothalamus and pituitary (Yin et al., 2005). The mammalian homolog of the GnIH receptor is GPR147 (OT7T022, NPFF1; Fukusumi et al. 2006), expressed in

the hypothalamus and pituitary (rodents and humans; Hinuma et al. 2000; Ubuka et al., 2009b). As a result of the location and function of this family of peptides and their receptor, an equine homolog of this peptide could play a role in reproductive seasonality in the equine by direct effects on the adenohypophysis.

2.8.1 RFRP3 hypothalamic expression, distribution, and function

Cellular *GnIH/RFRP3* expression distribution and fiber projections are variable between species and these differences may lend clues to the function of the GnIH/RFRP3 system in the mare. In the avian, cell bodies are located within the paraventricular nucleus of the hypothalamus (PVN), with GnIH-immunoreactive fiber projections most prominently extending towards the median eminence (Bentley et al., 2003; Ukena et al., 2003; Osugi et al., 2004), the site of GnRH exocytosis from neuronal terminals into the portal vasculature. Further, GnIH-containing fibers have been found in close proximity to GnRH neurons and fibers within the avian brain and GnRH-I and -II neurons express GnIH receptor (Bentley, 2003 2009). These observations indicate that GnIH may play both a central and peripheral role to regulate gonadotropin release in the avian. In the ovine, GnIH cell bodies are located within the dorsomedial nucleus of the hypothalamus (DMH) and PVN, with PVN cell bodies projecting towards the neurosecretory zone of the median eminence (Clarke et al., 2008). This supports the notion that RFRP3 acts directly at the level of the anterior pituitary to regulate gonadotropin release in the ovine (Clarke et al., 2008). In the equine, GnIH-immunoreactive neurons have been localized to the DMH with GnIH-containing fibers

in close proximity to GnRH dendrites and cell bodies as well as projecting towards the median eminence (Amstalden, Bentley and Williams, unpublished observations). However, RFRP3 hypothalamic distribution has not been evaluated in the equine, nor have differences in RFRP3 expression levels been observed across seasons - two components essential in deducing where in the equine reproductive axis RFRP3 is regulated.

2.8.2 Influence of melatonin on hypothalamic RFRP3 function

One factor potentially regulating mammalian hypothalamic GnIH/RPRP3 expression could be melatonin. Ubuka et al. (2005) demonstrated that GnIH-expressing cells also express the melatonin receptor (Mel1c) in the hypothalamus of avian species. Additionally, Gingerich et al. (2009) demonstrated a direct ability of melatonin infusion on immortalized hypothalamic cells to increase RFRP3 expression. Further, in the long-day breeding hamster, chronic infusion (60 d) of melatonin during the breeding season inhibited RFRP3 expression to non-breeding season levels (Revel et al., 2008). Therefore, GnIH/RFRP3 could serve as the intermediary signal between melatonin secretion and GnRH synthesis and secretion. If this is the case, melatonin or norepinephrine (a potent stimulator of melatonin biosynthesis) infusion during the natural breeding season should increase hypothalamic RFRP3 expression in the mare. Alternatively, infusion of cannabinoids to attenuate norepinephrine-induced melatonin biosynthesis (Koch et al., 2006) during the non-breeding season may release the mare from seasonal anovulation.

2.8.3 RFRP3 hypophyseal expression and function

The first vertebrate in which GnIH was isolated was Japanese quail and aptly named for its ability to inhibit adenohypophyseal synthesis and secretion of LH in a dose-dependent manner *in vitro* and *in vivo* (Tsutsui et al., 2000; Ubuka et al., 2006). Additional studies have illustrated a similar function of the mammalian homologue RFRP3 (Tsutsui et al., 2000; Kriegsfeld el al., 2006; Johnson et al., 2007; Clarke et al., 2008; Anderson et al., 2009) that functions through G protein-coupled receptor 147 (GPR147; alias NPFF1) expressed within the hypothalamus and pituitary (Hinuma et al. 2000, Ubuka et al., 2009b). Work in the ovariectomized female rat and ewe, as well as in pituitary cell cultures, indicates that RFRP3 plays a functional role at the level of the hypophysis to reduce gonadotropin responsiveness (Clarke et al., 2008; Murakami et al., 2008; Sari et al., 2009). This is further supported by evidence from anterograde tracing in the ewe that demonstrates RFRP3-immunoreactive cells project to the neurosecretory zone of the median eminence (Clarke et al., 2008). Further, reduced LHβ and FSHβ gene expression have been reported in GnRH-stimulated ovine pituitary cells treated with RFRP3 (Clarke et al., 2008). Interestingly, changes in FSHβ mRNA expression in pituitary cells collected from quail chronically treated with GnIH (Ubuka et al., 2006) and FSH levels in GnIH peripherally infused over 1 hr to ovariectomized ewes (Clarke et al., 2008) were not suppressed. Therefore, variation in GnIH/RFRP3 gonadotrope regulation exists between species and differences are dependent on treatment methods. Thus, it may prove fruitful to determine not only LHβ and FSHβ gene expression in equine gonadotropes treated both chronically and acutely with RFRP3, but also evaluate

differences in adenohypophyseal RFRP3 receptor (*GPR147*) gene expression across seasons to assess gonadotrope responsiveness as well as gonadotropin secretion in mares chronically infused with RFRP3. Alterations in one or more of these variables may be driving the seasonal reduction in ovarian activity that occurs during the non-breeding season in the mare.

In addition to genomic studies, determining if RFRP3 has a functional role at the level of the hypophysis will facilitate in determining the site of action for this peptide. As a 6- to 10-fold reduction in circulating LH concentrations are observed during the non-breeding season of the mare and recent results (Cooper et al., 2006) indicate that GnRH is not limiting during this time, it is reasonable to deduce that RFRP3 may be suppressing the responsiveness of gonadotropes to GnRH. Others have reported suppressed secretion of LH in pituitary cell cultures of male quail (Ubuka et al., 2006), rat (Murakami et al., 2008), chicken (Ciccone et al., 2004), and sheep (Clarke et al., 2008) treated with RFRP3. Interestingly, LH secretion was only suppressed in the presence of both GnIH/RFRP3 and GnRH. However, no reports have been published using equine pituitary cell culture to determine if RFRP3 infusion with or without GnRH suppresses LH secretion.

2.8.4 Peripheral and central RFRP3 infusion

Peripheral infusion of RFRP3 during the breeding and non-breeding seasons does indicate that that neither equine RFRP3 at any dose or manner tested, nor ovine RFRP3 as a large single dose, is able to suppress episodic release or mean concentration of LH

in mares (Prezotto, 2012). Further, treatment with RFRP3 was ineffective in modifying GnRH-induced release of LH in the mare (Prezotto, 2012) using doses similar to that reported previously in studies where RFRP3 suppressed LH in ovariectomized ewes (Clarke et al., 2008) and castrated male calves (Kadokawa et al., 2009). Thus, these reports fail to support the hypothesis that RFRP3 acts as an inhibitory signal to suppress the secretion of LH through effects at the hypothalamic or hypophyseal level in the mare. Similar results have been reported in ovariectomized pre-pubertal gilts (Heidorn et al., 2010) and ovariectomized, estradiol-replaced rats (Anderson et al., 2009) treated with RFRP3 intracerebroventricularly. However, ovariectomized female rats treated intravenously with RFRP3 concomitantly with GnRH exhibited reduced peak concentrations of LH when compared to controls (Rizwan et al., 2009). Therefore, functional differences within the RFRP3 system exist between species and are dependent on the dose, route of administration, and steroidal environment.

2.8.5 Role of RFRP3 during the breeding season

In addition to seasonal changes in RFRP3, this peptide may also function during the estrous cycle of the breeding season. Recent work by Magee et al. (2009) indicates that a similar peptide, kisspeptin, plays a role in regulating spring transition in the mare via up-regulation of LH gonadotrope function. However, this alone does not account for the 6 to 10-fold decline in LH associated with the transition from the ovulatory to anovulatory season. Furthermore, kisspeptin also has increased hypothalamic mRNA and protein levels under the influence of estradiol in female rats (Kinoshita et al., 2005),

while the role of estradiol in GnIH/RFRP3 function remains debatable (Kriegsfeld et al., 2006; Popa et al., 2008; Molnar et al., 2011). As the influence of estradiol in GnIH/RFRP3 function remains controversial, it is possible that the number of neurons expressing RFRP3 and cellular RFRP3 content in the hypothalamus of the mare could be elevated during the winter anovulatory period and luteal phase of the estrous cycle when photostimulation and circulating estradiol concentrations are minimized. Additionally, the role of the RFRP3 system in the mare could be gated down-stream of the mRNA level. Therefore, experiments designed to assess differences in RFRP3 translational rates as well as functional studies designed to evaluate vesicular exocytosis at neuronal terminals or receptor availability on target tissues may be necessary.

2.8.6 GPR147 expression, distribution, and function

Although RFRP3 appears to function through GPR147 expressed within both the hypothalamus and pituitary (Hinuma et al., 2000; Ubuka et al., 2009b), functional variability associated with RFRP3 effects has resulted in uncertainty regarding its mechanism of action in mammals. It is well documented that mammalian gonadotropes couple GnRH receptor with $G_{aq/11}$ to stimulate phospholipase C (PLC), resulting in the generation of inositol triphosphate (IP3) and diacylglycerol (DAG; Naor, 2009). These two components then activate protein kinase C (PKC) and trigger calcium mobilization to induce phosphorylation of mitogen-activated protein kinases (MAPKs; Naor, 2009). Mechanisms proposed in mammals to transmit GnIH/RFRP3 signal at the level of the gonadotrope ultimately result in inhibiting intracellular calcium mobilization (Clarke et

al., 2008), the primary GnRH receptor-induced signal for gonadotrope exocytosis (Stojilkovic and Catt, 1992; Stojilkovic and Catt, 1995), as well as phosphorylation of mitogen-activated protein kinases (ERK-1/2; Sari et al., 2009) to inhibit gonadotropin gene expression (Haisenleder et al., 1998). Therefore, GnIH/RFRP3 effectively reduces GnRH-induced gonadotropin synthesis and secretion. In the mare, data failed to demonstrate any functional effects of RFRP3 *in vivo* (Prezotto, 2012). However, recently, a selective antagonist for the NPFF1 receptor (RF9) has been shown to induce a potent release of LH in rats, mice, and sheep when administered centrally or peripherally (Pineda et al., 2010; Caraty et al., 2012; Rizwan et al., 2012), thus providing an additional approach for studying the RFRP3 system in the mare. The mechanism by which RF9 is functioning is unidentified. However, the possibility exists that the RFRP3 system acts directly on GnRH neurons. To date, all reported species (avian, rodents, ovine, sub-primates, humans, and equine) exhibit GnIH/RFRP3-immunoreactive fibers in close proximity to GnRH neurons (Bentley et al., 2003; Kriegsfeld et al., 2006; Smith et al., 2008; Ubuka et al., 2008; Qi et al., 2009; Ubuka et al., 2009a; Ubuka et al., 2009b; Amstalden, Williams, and Bentley, unpublished observation). Furthermore, GPR147 is expressed in GnRH neurons in birds (Ubuka et al., 2008) and rodents (Rizwan et al., 2012). Functional support for this hypothesis include the reduction in firing activity (Ducret et al., 2009; Ubuka et al., 2009b; Wu et al., 2009) and immediate early gene expression in GnRH neurons (Anderson et al., 2009) with administration of RFRP3. Furthermore, RFRP3/GnIH directly inhibits GnRH neurons by hyperpolarizing the

neuronal membrane through K+ conductance in dwarf gourami (Saito et al., 2010) allowing for the down-stream reduction in LH synthesis and secretion.

2.9 Expected impact of this research

The neuroendocrine control of reproductive seasonality is poorly characterized in the equine. Through investigations that address stimulatory and inhibitory mediators of the reproductive neuroendocrine axis of the mare, the equine industry will benefit at both a basic scientific and applied level. Using the information gathered from this research, the acceleration of reproductive transition from the winter anovulatory state and accelerated pregnancy may be possible using native GnRH. Additionally, experiments with RFRP3 may hold the key to an applied model to impede reproductive cyclicity in performance or feral horse populations or hasten reproductive cyclicity with the use of RFRP3 antagonists such as RF9. This expansion in the knowledge base of equine neuroendocrine function will be beneficial to our understanding of equine reproduction and to the equine industry.

CHAPTER III

THERAPEUTIC APPLICATION OF NATIVE GNRH IN THE WINTER ANOVULATORY MARE, I: FREQUENCY OF REVERSION TO THE ANOVULATORY STATE FOLLOWING OVULATION INDUCTION AND CESSATION OF TREATMENT

3.1 Synopsis

The continuous administration of native GnRH to seasonally anovulatory mares stimulates successfully the synthesis and secretion of LH without pituitary refractoriness. Herein we tested the hypothesis that continuous administration of native GnRH, beginning in either early February or early March (North America), will rapidly stimulate follicular development and ovulation without resulting in reversion of cyclic mares to an anovulatory state following treatment withdrawal. Anovulatory mares received sham pumps (Control) or native GnRH (100 μg/h) for 28 d beginning either February 2 or 3 (GnRH-Feb) or March 2 or 3 (GnRH-Mar). Mean concentrations of LH were 5 to 7-fold greater in GnRH-Feb compared to control and GnRH-Mar mares through March 2 or 3. However, concentrations of LH returned to the winter baseline within 3 to 11 d after pump removal and all GnRH-Feb mares failed to remain cyclic following treatment withdrawal. Correspondingly, concentrations of LH in GnRH-Mar mares were greater ($P < 0.001$) than in control and GnRH-Feb mares for the 28-d period; however, ovulatory cycles continued in all mares after cessation of treatment. Follicular growth and frequency of spontaneous ovulation (6/10 GnRH-Feb; 9/10 GnRH-Mar, 1/11

controls, respectively) was greater ($P < 0.01$) in GnRH-treated mares. Continuous administration of native GnRH for 28 d, beginning in early February or March, elevated circulating LH adequately to stimulate follicular growth and spontaneous ovulation up to 60 d earlier than in untreated controls. However, if continuous GnRH treatment is selected as the only therapeutic intervention, and mares are not pregnant, GnRH treatment must continue through March in order to ensure continued escape from the winter anovulatory state.

3.2 Introduction

The seasonal termination of ovarian cycles in mares, a process initiated near the time of the autumnal equinox and completed in most of the mare population by the winter solstice, is a significant managerial issue for professional horse breeders world-wide (Ginther, 1992; Davies, 2003). Currently, the provision of supplemental lighting for at least 3 mo is the only practical intervention strategy available, and offers the ability to advance the operational breeding season by approximately 1 mo (Burkhart, 1947; Ginther, 1992; Davies, 2003).

From an endocrine perspective, one of the most conspicuous features associated with the winter anovulatory period in the mare is the marked decline in anterior pituitary synthesis and secretion of LH (Garcia and Ginther, 1975; Turner et al., 1979; Hart et al., 1984). Johnson (1987) demonstrated unequivocally that hourly, very low-dose (2 μg) intravenous pulses of native GnRH could quickly increase circulating concentrations of LH, stimulate follicular growth, and initiate spontaneous ovulation in mares in a state of

deep winter anovulation. Importantly, a lesser but highly-effective pituitary response was noted with continuous administration of GnRH in a large-dose (100 μg/h; Becker and Johnson, 1992). These and other studies revealed that, unlike other female mammals (Belchetz et al., 1978; Knobil, 1981), an intermittent hypophysiotropic signal is not required for the equine GnRH receptor to remain functionally responsive (Johnson, 1987; Hyland et al., 1987; Porter et al., 1997). Thus, unique opportunities for practical intervention strategies using GnRH seemed inevitable.

Experiments with potent GnRH agonists (GnRHa), injected twice daily (Ginther and Bergfelt, 1990; Harrison et al., 1990; McCue et al., 1991; McCue et al., 1992) or administered as an implant or depot formulation soon followed (Harrison et al., 1990; Fitzgerald et al., 1993; Mumford et al., 1994). McCue et al. (1992) demonstrated efficacy of twice daily injections of GnRHa (buserelin) for achieving pregnancy with mares in a deep anovulatory state. Unfortunately, the more practical, continuous delivery of similar GnRHa resulted in low or inconsistent responses and evidence of pituitary desensitization (Harrison et al., 1990; Fitzgerald et al., 1993; Mumford et al., 1994). Hence, available evidence supports the practical superiority of continuously-applied, native GnRH, as opposed to GnRHa, for initiating follicular development and ovulation in mares in the middle of the non-breeding season (Hyland et al., 1987; Johnson, 1987; Becker and Johnson, 1992), and for accelerating the timing of pregnancy of mares in late transition (Hyland et al., 1987; Hyland and Jeffcott, 1988). Unfortunately, this paradigm has not been tested for its ability to accelerate the timing of pregnancy in mares in deep,

winter anovulation, nor is there a commercially-approved delivery platform available for this or related purposes.

Our long-term objectives are to develop the physiological and managerial principals needed to apply GnRH therapeutics for routinely establishing pregnancies in the winter anovulatory mare. In the experiments described here, the primary interest was to determine whether anovulatory mares treated continuously for 28 d with native GnRH, beginning in either early February or early March (Northern Hemisphere), will establish normal luteal function following spontaneous ovulation and remain cyclic following treatment withdrawal.

3.3 Materials and methods

The Institutional Animal Care and Use Committee of Texas A&M University approved in advance all procedures used in this study.

3.3.1 Animals and diets

Thirty-one light horse mares, predominantly American Quarter Horses, were maintained on a pasture of mixed grasses and supplemented with coastal Bermuda grass hay and a pelleted concentrate (12% CP) as needed to maintain an average body condition score (BCS) of 5 (1 to 9 scale; Henneke et al., 1983). Mare BCS was assessed before treatment onset and monthly for the duration of the study. Mean BCS and age of the mares used in this study were 4.9 ± 0.3 and 9.3 ± 1.8, respectively. Mares had continual access to water and trace mineralized salt throughout the study.

3.3.2 Treatments and experimental design

Beginning the first week of January and continuing for the next 3 wk, ovulatory status of mares was determined using transrectal ultrasonography (Honda HS-Feb500V, Honda Electronics Co., LTD) combined with serum concentrations of progesterone determined in jugular blood samples collected once weekly. Mares were confirmed as anovulatory based on the absence of a corpus luteum (CL), no follicle > 28 mm, and serum progesterone at baseline (~ 0.1 ng/mL) during this period. However, 6 of 31 mares had follicles > 20 mm at the start of the experiment. Mares were stratified by age, starting BCS, and size of largest follicle and assigned randomly to 1 of 3 groups: 1) Control (n=11), 2) GnRH-Feb (n=10), or 3) GnRH-Mar (n=10). As the same mares were used as controls for GnRH-Feb and GnRH-Mar, one additional Control mare was included to account for potential spontaneous ovulation in this group. Thus, Control mares were classified as Control-Feb or Control-Mar during the 28-d treatment periods in February and March, respectively. When differences did not exist between Control-Feb and Control-Mar, treatments were pooled for analysis. Treatments or sham treatments were initiated in each experimental group over 2 consecutive d on February 2 or 3 and March 2 or 3. On February 2 or 3, mares assigned to GnRH-Feb were fitted surgically with subcutaneous Alzet osmotic pumps (model 2ML2; Durect Corp, Cupertino, CA) to deliver GnRH at a rate of 100 μg/h for 14 d. Control and GnRH-Mar mares were fitted similarly with sham pumps constructed of Silastic tubing (Dow Corning Corporation, Midland, MI) filled with medical grade silicone adhesive (Dow Corning Corporation, Midland, MI). On February 16 and 17, respectively, the initial 14-

d pumps (GnRH-Feb) and sham pumps (control and GnRH-Mar) were removed and similar new pumps/sham pumps were installed. This same process was repeated in March except that GnRH-Mar mares received Alzet osmotic pumps, and control and GnRH-Feb mares received sham pumps. Figure 3.1 provides a time-line of the experiment.

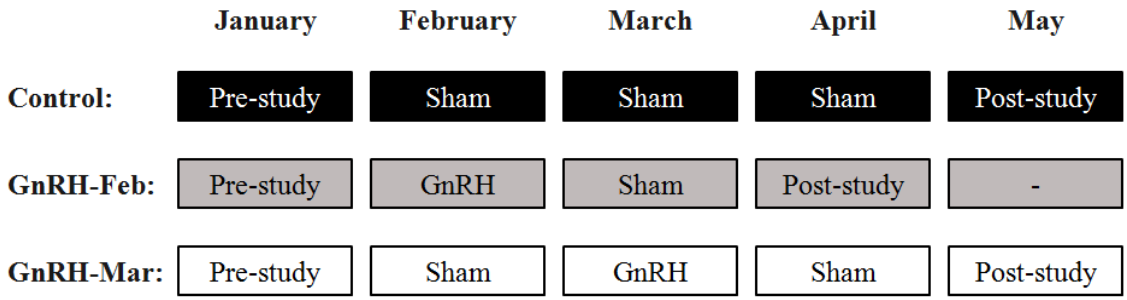

Fig. 3.1. Experimental time line depicting the treatments applied from January to May in control, GnRH-Feb, and GnRH-Mar treated mares. All mares were confirmed anovulatory utilizing transrectal ultrasonography and confirmation of serum concentrations of progesterone at baseline during the Pre-study period in January. Continuous, subcutaneous GnRH treatments were applied in either February or March. When not treated with GnRH, mares received sham pumps fitted subcutaneously followed by a Post-study period where blood was collected to monitor ovarian activity.

3.3.3 Subcutaneous placement of pumps

In accordance with manufacturer's recommendations, Alzet pumps were incubated in physiological saline (0.9% NaCl solution) at 37ºC for 16 h prior to implantation. Mares were placed in a stock and sedated with detomidine hydrochloride (Dormosedan; 20-40 µg/kg BW; Pfizer Animal Health, New York, NY) if required. An area at the base of the neck (anterior to the scapula) was clipped and prepared for aseptic surgery using an iodophore (Purdue Product LP, Stamford, CT), povidone iodine

solution (Prepodyne, WestAgro, Kansas, MO), and 70% isopropanol. Three to five milliliters of a local anesthetic (lidocaine HCl, 2%; Agri Laboratories, St. Joseph, MO) was injected subcutaneously and a 2-cm incision was made through the skin with a sterile scalpel. A sterile, blunt instrument was used to expand the subcutaneous space making a pocket to accommodate the GnRH or sham pump. The incision was closed with non-absorbable synthetic sutures that were left in place for 7 d. At the end of each 14-d period, Alzet pumps or sham pumps were removed and replaced using a similar procedure. Each consecutive 14-d device was applied at a different location by alternating between each side of the neck. Evacuated subcutaneous pockets were flushed with dilute povidone iodine solution followed by surgical closure with 2 to 3 synthetic sutures (Nylon, # 2). Implantation sites were monitored daily and, if inflammation or infection was observed, the skin over the surgical site was cleaned, disinfected with povidone iodine, and an antibacterial ointment applied. Only minor inflammatory changes were noted during this study.

3.3.4 Estrous behavior

Mares were teased daily in a large pen of 5 to 6 mares using an intact stallion in an adjacent pen separated by a solid panel. Teasing scores were assessed utilized a scale ranging from 1 to 4 (1, breaking down in the presence of the stallion; 2, winking in the presence of the stallion; 3, indifference to the stallion; 4, physical rejection evidenced by kicking, and ear-pinning).

3.3.5 Ultrasonography

Transrectal ultrasonography was performed every 3 d beginning at onset of the study. When a follicle ≥ 30 mm was detected, ultrasound examinations were performed daily until ovulation was confirmed and continued for 30 d following cessation of each 28-d treatment period.

3.3.6 Blood collection

Single blood samples were collected via jugular venipuncture at the following intervals: once weekly for four weeks (pre-trial), every other day for eight weeks (28-d treatment-period and 28-d follow up-period), and twice weekly for four weeks (post-study) for determination of serum concentrations of progesterone and LH. Samples were placed on ice immediately for transport to the laboratory, kept under refrigeration at 4º C for approximately 1 h, and then allowed to clot at room temperature for approximately 45 min prior to centrifugation. Samples were centrifuged at 4°C, with serum harvested and stored at -20°C until analysis.

3.3.7 Hormone analyses

Serum concentrations of LH were assayed in duplicate utilizing a double antibody RIA as validated previously in this laboratory (Williams et al., 2007). A highly purified equine LH (LH AFP-5130A) was used for both iodinated tracer and standards. An anti-equine LH antiserum (AFP-240580) was used at a dilution of 1:100,000. Serum concentrations of progesterone were assayed using a commercial kit (Diagnostic

Products Corporation, Los Angeles, CA) using a single-antibody coated tube RIA. Assay sensitivities were 0.1 ng/mL for both LH and progesterone. Inter- and intra-assay CV for the LH assay averaged 6.1 and 8.5%, respectively. Inter-and intra-assay CV for the progesterone assay averaged 4.2 and 7.2%, respectively.

3.3.8 Statistical analysis

Analysis of repeated measures was used to determine main effects of treatment on temporal changes in serum hormone concentration and estrous behavior utilizing a mixed model procedure (PROC MIXED) of the Statistical Analysis System (SAS). The model contained fixed effects for treatment, block, day, and the treatment x day interaction. The variable 'day' served as the repeated variable and 'mare' as the subject. Serum hormone concentrations and estrous behavior were also analyzed after normalization to onset of GnRH treatment (Feb 2 or 3 and March 2 or 3). The PROC GLM procedure of SAS was used to determine main effects of treatment on interval to ovulation, interval to declines in concentrations of LH post-ovulation and post-treatment withdrawal, interval from treatment withdrawal to baseline concentrations of LH, and change in follicle size during the 28-d treatment periods. The model contained fixed effects for treatment, and block. Comparisons were also made for mares that responded (ovulated) to GnRH treatments in February (n = 6) and March (n = 9). Enumerative data were analyzed using the FREQ procedure (PROC FREQ) of SAS and the Fisher's Exact Test. Main effects were considered significant when $P \leq 0.05$ and a trend toward

significance when $P \leq 0.10$. Upon detection of a significant F-test, LS means were contrasted by the method of least significant difference.

3.4 Results

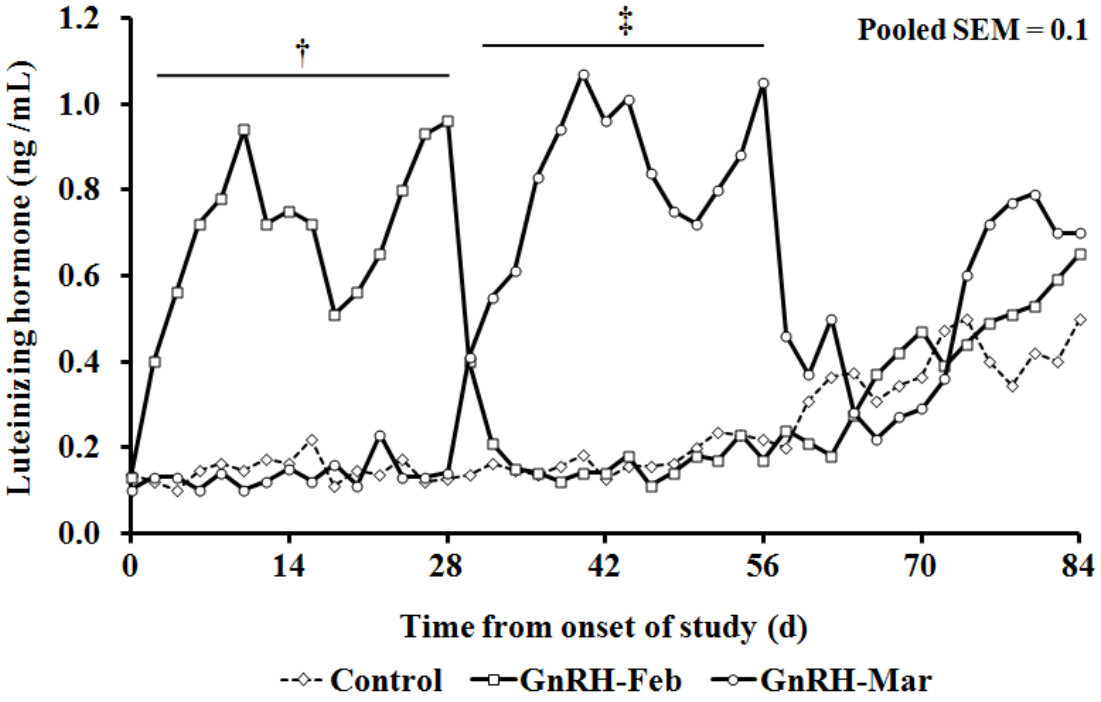

Fig. 3.2. Least squares mean concentrations of LH during February, March and April in which mares served as controls (n = 11) or were treated with GnRH (100 µg/h) continuously for 28 d beginning either on February 2 or 3 (GnRH-Feb; n= 10) or March 2 or 3 (GnRH-Mar; n = 10). †Denotes a difference ($P \leq 0.02$) between control and GnRH-Mar mares from GnRH-Feb mares. ‡Denotes a difference ($P \leq 0.02$) between control and GnRH-Feb mares from GnRH-Mar mares. Treatment x day $P < 0.0001$.

3.4.1 Serum LH

Mean concentrations of LH did not differ among groups at the start of the experiment on February 2 and 3; however, within 48 h and throughout the remainder of

the 28-d treatment period, serum concentrations of LH were greater ($P \leq 0.02$) for GnRH-Feb mares than in both Control-Feb and GnRH-Mar mares (Fig. 3.2). Removal of GnRH pumps in GnRH-Feb mares resulted in a decline in circulating LH, returning to pre-treatment values and not different from Control-Feb and GnRH-Mar-treated mares. Similarly, serum concentrations of LH in GnRH-Mar-treated mares increased rapidly within 48 h of treatment onset (March 2-3), with mean concentrations greater ($P \leq 0.02$) than both Control-Mar and GnRH-Feb mares throughout the 28-d March treatment period (Fig. 3.2). When serum LH was normalized to the onset of GnRH or sham treatments in all groups (Fig. 3.3), mean concentrations of LH were greater ($P \leq 0.02$) on treatment days 12 and18 in GnRH-Mar compared to GnRH-Feb mares. Following GnRH treatment withdrawal at the end of March, mean serum concentrations of LH dropped initially and then rebounded in association with a continuation of ovarian cyclicity in all GnRH-Mar mares that had spontaneously ovulated. During this same period (April), serum LH in control and GnRH-Feb mares was also increasing in concert with onset of the natural breeding season; thus, mean concentrations were similar among all groups at this time (Fig. 3.2).

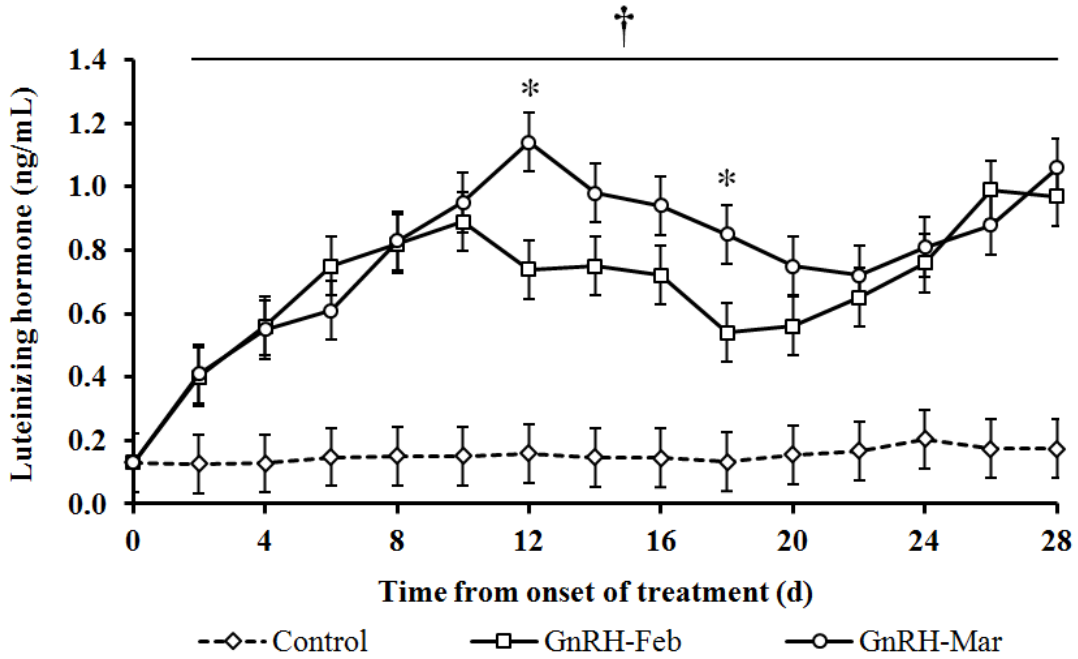

Fig. 3.3. Least squares mean concentrations of LH, normalized to the day of onset of treatments, in which mares served as controls (n = 11) or were treated with GnRH (100 µg/h) continuously for 28 d beginning either on February 2 or 3 (GnRH-Feb; n= 10) or March 2 or 3 (GnRH-Mar; n = 10). *Denote a difference ($P \leq 0.02$) between GnRH-Feb and GnRH-Mar treated mares. †Denote a difference ($P \leq 0.02$) between GnRH-treated and control mares. Treatment x day $P < 0.0001$.

During both the February and March 28-d treatment periods, a biphasic pattern in mean serum concentrations of LH was observed. Initial mean peaks occurred on treatment days 10 and 12 in GnRH-Feb and GnRH-Mar-treated mares, followed by a second peak on treatment day 28 (Fig. 3.2). Individual mares exhibited declines in concentrations of LH from 0 to 10 d post-ovulation, with mean intervals (GnRH-Feb 3.3 ± 1.2 d; GnRH-Mar 3.8 ± 1.0 d) not different ($P = 0.78$) between GnRH treatments. Declines in circulating concentrations of LH also occurred between 3 and 8 d post-treatment withdrawal, with mean intervals of 3.2 ± 0.3 d and 3.4 ± 0.3 d not different (*P*

= 0.66) for GnRH-Feb and GnRH-Mar, respectively. All GnRH-treated mares, excluding two GnRH-Mar mares, returned to baseline (0.1 ng/mL) concentrations of LH between 3 and 11 d post-treatment withdrawal. However, interval from treatment withdrawal to baseline did not differ ($P = 0.33$) between GnRH-Feb (6.2 ± 0.7 d) and GnRH-Mar (7.3 ± 0.8 d).

3.4.2 Ovarian morphology, ovulatory responses, and serum progesterone

Mares treated with GnRH (GnRH-Feb and GnRH-Mar) had increased ($P \leq 0.01$) follicular growth during the 28-d treatment period than controls (Table 3.1). The occurrence of spontaneous ovulation was greater ($P \leq 0.01$) in GnRH treated mares compared to control mares with 6 of 10 GnRH-Feb and 9 of 10 GnRH-Mar treated mares ovulating spontaneously during their respective treatment periods compared to only 1 of 11 Control mares which ovulated during March (Fig. 3.4). Therefore, intervals to first ovulation for both GnRH-Feb and GnRH-Mar were decreased ($P \leq 0.001$; Table 3.2) relative to controls. For mares that ovulated during each of the respective treatment periods (GnRH-Feb: 6 of 10 and GnRH-Mar: 9 of 10), mean intervals to ovulation was 14.0 ± 4.5 and 11.7 ± 3.7 d for GnRH-Feb and GnRH-Mar, respectively.

Table 3.1. Least squares mean (± SEM) maximal change in follicle size (mm) during the three 28-d treatment intervals. Mare treatments were control: sham pump surgically implanted subcutaneously for 28 d during February and March (n = 11); GnRH-Feb: GnRH delivered subcutaneously in saline at a rate of 100 µg/h for 28 d during February (two consecutive Alzet 2ML2; n = 10); and GnRH-Mar: GnRH delivered subcutaneously in saline at a rate of 100 µg/h for 28 d during March (two consecutive Alzet 2ML2; n = 10). [a,b]Means without a common superscript letter differ ($P \leq 0.01$) within treatment interval.

	Treatment interval (d)		
Treatment	**0 - 28**	**28 - 56**	**56 - 84**
Control	8.7 ± 2.0^{a}	7.5 ± 2.5^{a}	18.9 ± 3.2^{a}
GnRH-Feb	19.6 ± 2.1^{b}	8.5 ± 2.6^{a}	-
GnRH-Mar	-	19.2 ± 2.6^{b}	23.1 ± 3.4^{a}

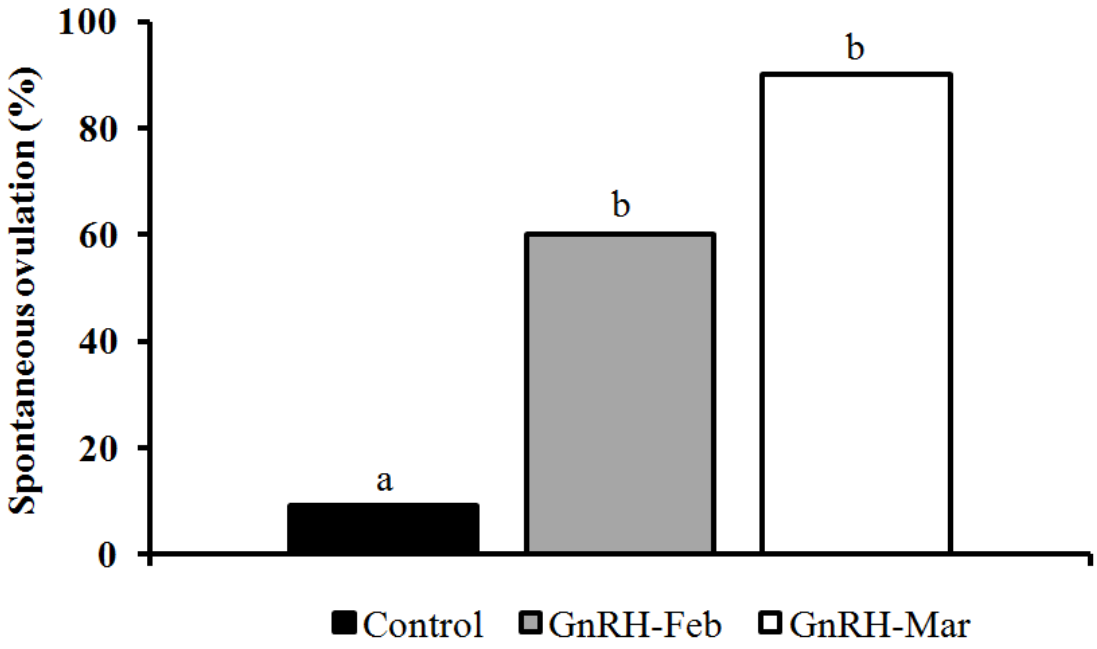

Fig. 3.4. Proportion of mares that ovulated spontaneously within their respective 28-d treatment periods (GnRH-Feb and GnRH-Mar) compared to control mares during the same 28-d periods pooled. Proportions without a common superscript differ ($P \leq 0.01$).

Table 3.2. Least squares mean (± SEM) interval (d) from onset of treatment to first ovulation normalized to onset of treatment. Mare treatments were control: sham pump surgically implanted subcutaneously for 28 d during February and March (n = 11); GnRH-Feb: GnRH delivered subcutaneously in saline at a rate of 100 μg/h for 28 d during February (two consecutive Alzet 2ML2; n = 10); and GnRH-Mar: GnRH delivered subcutaneously in saline at a rate of 100 μg/h for 28 d during March (two consecutive Alzet 2ML2; n = 10). [a,b,c]Means without a common superscript letter differ ($P \leq 0.01$) within parameter. [1]Denotes the inclusion of all GnRH-treated mares (GnRH-Feb n = 10; GnRH-Mar n = 10). [2]Denotes the removal of GnRH-treated mares that failed to ovulate within the 28-d GnRH treatment period (GnRH-Feb n = 6; GnRH-Mar n = 9).

	Interval to first ovulation (d)	
Treatment	**All[1]**	**Responsive[2]**
Control	75.8 ± 6.1[a]	-
GnRH-Feb	38.4 ± 6.4[b]	14.0 ± 4.5[a]
GnRH-Mar	16.7 ± 6.4[c]	11.7 ± 3.7[a]

All mares exhibited progesterone concentrations < 1 ng/mL for the 3-wk pre-trial period leading up to the experiment on February 2. Since blood samples were collected every other day during the experimental treatment periods and not relative to day of induced cycles, statistical contrasts of circulating progesterone are not relevant. However, all mares that ovulated during the 28 d treatment periods exhibited morphologically normal-appearing CL and typical patterns of progesterone secretion (Fig. 3.5).

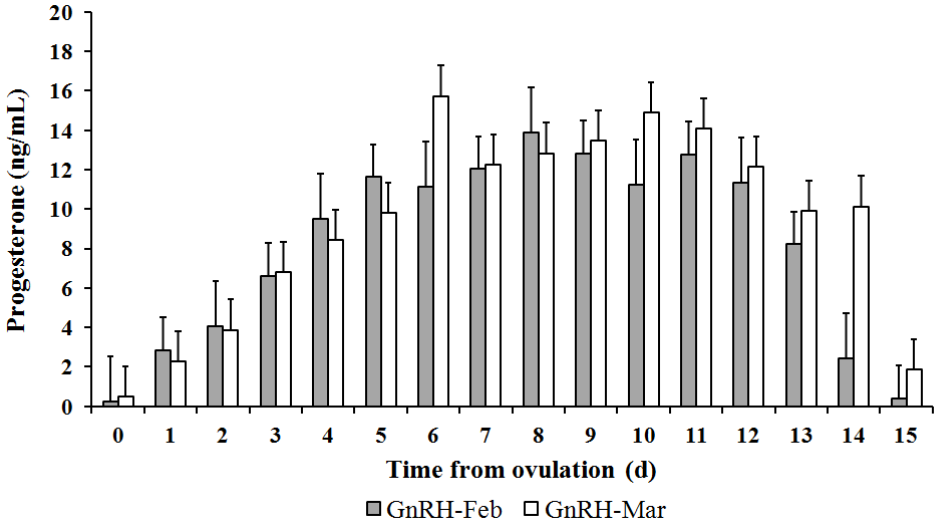

Fig. 3.5. Least squares mean (± SEM) concentrations of progesterone during the 14-d period following ovulation in GnRH-Feb (n = 6) and GnRH-Mar (n = 9) mares.

Mares treated with native GnRH in March and responsive to treatment (ovulated), continued to cycle following treatment withdrawal and exhibited two (n = 3) or three (n = 6) follicular waves by the end of May with corresponding increases in circulating concentrations of progesterone. Further, all mares that failed to ovulate within the 28-d treatment period (February n = 4; March n = 1) had elevated serum progesterone concentrations (peak progesterone ranging from 5.8 to 15.1 ng/mL) within 60 days of treatment withdrawal and thus possess a functional hypothalamic-pituitary-ovarian axis.

3.4.3 Teasing scores

Between days 3 to 16 and days 3 to 15 following initiation of treatments in GnRH-Feb and GnRH-Mar mares, respectively, the number of mares exhibiting estrus

was greater and thus mean teasing scores were lower ($P \leq 0.01$) than in control mares (Fig. 3.6). GnRH-treated mares began exhibiting consecutive days of estrous behavior, equal to a teasing score of 1, between 3 and 16 d prior to ovulation. Mean duration of consecutive days of estrous behavior (teasing scores equal to 1) were 8.8 ± 1.5 d and 6.4 ± 1.2 d for GnRH-Feb and GnRH-Mar, respectively. However, duration of estrous behavior was not different ($P = 0.23$) between GnRH treatments and all GnRH-treated mares demonstrated estrous behavior on the day of ovulation.

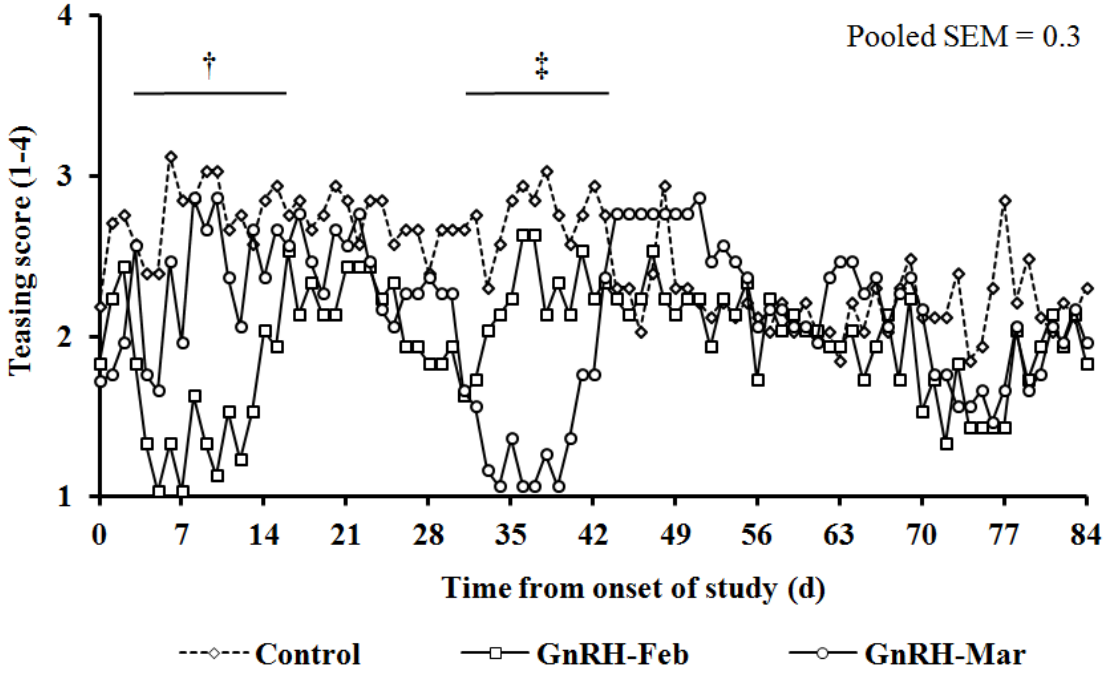

Fig. 3.6. Least squares mean teasing scores in control, GnRH-Feb, and GnRH-Mar groups. Individual scores (1 = mare breaking-down in the presence of the stallion to 4 = mare rejecting the stallion) were assigned daily. †Denotes a difference ($P \leq 0.01$) between GnRH-Feb treated mares and control. ‡Denotes a difference ($P \leq 0.01$) between GnRH-Mar treated mares and controls. Treatment x day; $P < 0.0001$.

3.5 Discussion

Results of the current study confirm that continuous treatment with native GnRH is an effective means of initiating ovulatory cycles beginning as early as 2 mo before the average onset of the natural breeding season (Johnson, 1987; Becker & Johnson, 1992). However, under the conditions of this study, in which ovulations were allowed to occur spontaneously, it appears that mares will not continue to exhibit estrous cycles following termination of treatment on March 2 or 3. To the contrary, when treatments are initiated on March 2 or 3 and terminated in late March, ovulatory cycles continue. The basis of this observation is likely the lack of adequate endogenous secretion of GnRH in February and its subsequent increase as mares transition into the natural breeding season in late March (Sharp and Grubaugh, 1987). Thus, regardless of whether treatment is initiated in February or March, it appears that it must be continued to the end of March if applied as in the current experiment. However, based on two other published studies, it is possible that continuous treatment through March may not be required if the preovulatory follicle is induced to ovulate using an exogenous gonadotropin or a gonadotropin surge-inducing treatment. In one study (Bergfelt and Ginther, 1992), mares with minimal ovarian activity at initiation of GnRH treatment were shown to be more likely to develop subnormal luteal function following spontaneous ovulation. In contrast, McCue et al (1992), using twice daily injections of buserelin to stimulate follicular development in mares in deep winter anovulation, observed high pregnancy rates (> 70%) and low (7%) embryonic losses when preovulatory follicles were induced to ovulate using hCG. Therefore, subnormal luteal function, increased risk of embryonic

loss, and failure to continue to ovulate (non-pregnant mares) may potentially be avoided after termination of continuous GnRH treatment if the first ovulation is induced using hCG or an ovulatory dose of a surge-inducing treatment with GnRH or GnRHa.

In association with the above observations, and as expected, there was a steady increase in circulating LH in response to GnRH treatments in both months. Mares treated with GnRH at a rate of 100 µg/h for 28 d exhibited a 5 to 7-fold increase in circulating LH within 2 wk, further substantiating the well-established thesis that continuous infusion of native GnRH in the mare does not cause overt GnRH receptor down-regulation as it does in other species (Belchetz et al., 1978; Porter et al., 1997). In addition, the mean treatment response to continuous GnRH treatment was biphasic. Peaks in mean concentrations of LH were observed on treatment days 10 and 12 and again on day 28 for both GnRH-Feb and GnRH-Mar groups. Upon evaluation of concentrations of LH for individual mares, the initial peak appears to be associated with the occurrence of falling concentrations of LH post-ovulation in a majority of the mares during each treatment month. The progressive decline in circulating concentrations of LH occurring 2 to 7 d post-ovulation is well documented during the natural breeding season (Evans and Irvine, 1975; Geschwind et al., 1975; Noden et al., 1975; Pattison et al., 1975) and appear to be consistent with observations in the current study. The descending aspect of the second peak was likely a function of treatment withdrawal in each group. A similar pattern of response to continuous GnRH treatments in the mare was reported by Hyland et al. (1987). However, in that study, peaks were observed on days 7 and 21 with intervals to first ovulation averaging 19.9 ± 1.7 d. Therefore,

ovulation was associated with the second peak in circulating LH in that study. This differential response may potentially be accounted for by the greater dose of GnRH given in the current study (100 μg/h) compared to the previous work (~ 40-60 μg/h). In addition, the minimum peak concentration of LH sufficient to induce ovulation during February was 0.7 ng/mL in responsive mares; however, non-responsive mares also exhibited peak concentrations of LH between 0.8 and 1.8 ng/mL. Thus, the minimum threshold concentration of LH needed to develop a spontaneously-ovulatory follicle in the winter anovulatory mare appears to vary. As noted below, this did not appear to be related to the size of the largest follicle at the onset of treatment in the current experiment.

The GnRH treatments used in the current work induced follicular growth and markedly accelerated timing of first spontaneous ovulation in 15 of 20 (75%) treated mares during February or March, compared to only 1 of 11control mares during the same interval. These ovulatory responses were similar to those reported by others using both native GnRH (Johnson, 1987; Hyland and Jeffcott, 1988; Becker and Johnson, 1992; Thorson et al., 2011) and GnRH agonists (Bergfelt and Ginther, 1992; McCue et al., 1992; Fitzgerald et al., 1993; Mumford et al., 1994), and intervals to first ovulation were comparable to that reported previously (Allen et al., 1987; Hyland et al., 1987; Hyland and Jeffcott, 1988; McCue et al., 1992). However, this is the first study to show that winter anovulatory mares, if treated only in February (North America), do not continue to exhibit ovarian cycles following treatment withdrawal when allowed to

ovulate spontaneously. In contrast, if mares were treated through March, ovarian cyclicity persisted.

Ovarian responsiveness of mares to GnRH treatment did not appear to differ appreciably between mares having follicles lesser or greater than 20 mm in diameter, and agree with an accompanying study (Thorson et al., 2011). Other reports have indicated that size of the largest follicle at treatment onset does have a major influence on timing and frequency of ovarian responses (Ginther and Bergfelt, 1990; Bergfelt and Ginther, 1992). This may be related directly to the concentrations of LH achieved in the peripheral circulation in response to various treatments, some of which have been much less effective than that described here and in other reports (Johnson, 1987; Becker and Johnson, 1992). In spite of the relatively high concentrations of LH induced by GnRH-treatments in the winter anovulatory mare, multiple ovulations were not observed in GnRH-treated mares in our study. However, multiple ovulations has been reported in response to potent GnRHa by others during the winter anovulatory season (McCue et al., 1992), spring transition (Ginther and Bergfelt, 1990; Raz et al., 2009), and breeding season (Fitzgerald et al., 1993). Treatment with native hormone has also been shown to increase the rate of multiple ovulation during the anovulatory season but only when delivered intravenously in a continuous or pulsatile manner (Johnson and Becker, 1988; Becker and Johnson, 1992) and not when administered subcutaneously (Velez et al., 2008; Thorson et al., 2011). The basis of these differing results is not clear. Interestingly, Ginther and Bergfelt (1990) reported a greater frequency of multiple ovulations (64%) when the largest follicle present at the time of treatment onset was larger than 25 mm,

whereas Raz et al. (2009) observed only a 7% incidence of multiple ovulations when GnRH treatment was initiated in mares bearing follicles greater than 25mm. Breed type appears also to play a significant role in the rate of multiple ovulations, with Quarter Horse mares exhibiting a less frequent incidence than Thoroughbred mares (Ginther, 1982). In the current work, the majority of mares were Quarter Horse or grade mares.

Duration of estrus behavior ranges from 2 to 12 d for individual mares, with mean days in estrus during February (6.7 d) and March (8.4 d) greater than that observed during the natural breeding season (April-October, 5.2 d; Hughes et al., 1972; Ginther, 1992). Varying degrees of teasing behavior in this study increased markedly soon after the onset of GnRH treatments in both February and March-treated mares, occurring well in advance of the presence of a large follicle. This phenomenon has been observed before (Thorson et al., 2011) and may be a function of heightened sensitivity to ovarian steroids (estradiol, androgens) during the anovulatory period.

Johnson and Becker (1988) reported measurable levels of estradiol within 3 to 7 d of beginning pulsatile GnRH infusion in the winter anovulatory mare, observing mean peak concentrations of estradiol (20 ± 2 pg/mL) similar to peak concentrations observed during the breeding season (20 ± 3 pg/mL; Daels et al., 1991). Furthermore, concentrations of estradiol in mares treated with GnRH in a pulsatile fashion were greater than mean peak concentrations observed during the winter anovulatory (1.3 ± 0.3 pg/mL), transitional (2.0 ± 0.5 pg/mL), and preovulatory period of the first vernal transition ovulation (6.3 ± 0.7 pg/mL) of non-GnRH-treated mares (Donadeu and Ginther, 2002). In contrast, Hyland et al (1987) reported that despite the occurrence of

normal ovulations and formation of normal CL, estradiol concentrations remained undetectable throughout a 28-d continuous GnRH treatment period. Another possibility for precocious estrus behavior is heightened androgen production by small follicles, but this is purely speculative and has not been tested. Further, all mares in the current experiment that developed a large follicle and ovulated in response to GnRH treatment exhibited full expression of estrous behavior, equivalent to a teasing score of 1, during the periovulatory period. This finding is indicative of steroidogenically-competent ovarian follicles that are potentially fertile as reported elsewhere (Bergfelt & Ginther, 1992; McCue et al., 1992; Thorson et al., 2011).

In conclusion, the current study indicates that the robustness of anterior pituitary and ovarian responses of winter anovulatory mares to continuous GnRH treatments are similar whether initiated in early February or March in North America. However, since strategies to accelerate Julian day foaling dates are the primary concern of breeders, it would seem plausible to begin such treatments in early February rather than in March. If this approach is chosen and mares are allowed to ovulate spontaneously, without any additional gonadotropic support, it appears that continuous GnRH therapy would need to continue to the end of March in order to assure continued cyclicity in the absence of pregnancy. However, prevailing evidence indicates that continued treatment following ovulation may not be necessary (McCue et al., 1992) if mares are induced to ovulate with additional gonadotropic support (e.g., hCG or surge-inducing treatment with GnRHa). Such a strategy would help to ensure normal luteal function and thus maintenance of pregnancy if mares are bred. In an accompanying study (Thorson et al.,

2011), continuous GnRH treatment through March was used successfully in conjunction with hCG-induced ovulation. However, more direct tests are needed to determine whether continuous GnRH treatment can be terminated in a normal management scenario if ovulations are induced as described above as opposed to being allowed to occur spontaneously. Nonetheless, in spite of the biological advantage of native GnRH in this setting, as shown in the current and accompanying report (Thorson et al., 2011), its value continues to remain unexploited from a commercial perspective. This appears to be driven mainly by the current lack of a practical and efficient delivery platform that does not require minor surgery (e.g., injectable depot delivery). This laboratory is in the process of investigating the development of such an approach.

CHAPTER IV

THERAPEUTIC APPLICATION OF NATIVE GNRH IN THE WINTER ANOVULATORY MARE, II: ACCELERATING THE TIMING OF PREGNANCY

4.1 Synopsis

Onset of the winter anovulatory period in mares is associated with a marked diminution in adenohypophyseal synthesis and release of LH. Native GnRH, unlike its synthetic agonists, stimulates the synthesis and secretion of LH in mares without pituitary refractoriness. Herein we tested the hypotheses that 1) the average Julian day of pregnancy can be accelerated by up to 2 mo in winter anovulatory mares treated continuously with native GnRH beginning on February 1 and 2) mares will sustain luteal function and pregnancy following treatment withdrawal. Forty-two winter anovulatory mares were stratified by age, BCS, and size of largest follicle across two locations in a randomized design and assigned to 1 of 3 groups (n = 14/group): 1) Control: untreated; 2) GnRH-14: GnRH delivered subcutaneously in saline at a rate of 100 μg/h for 8 wk (Feb. 1–Mar. 29) using 4 consecutive 14-d pumps (Alzet 2ML2), or 3) GnRH-28: GnRH delivered as in 2, but using two, 28-d pumps (Alzet 2ML4). Upon development of a 35 mm follicle and expression of estrus, mares were bred the following day and treated with hCG. Pregnancies were confirmed by transrectal ultrasonography on days 14, 24, 33, and 45, with blood samples collected to assess luteal function. Mares treated with GnRH (GnRH-14 and GnRH-28) exhibited marked increases ($P \leq 0.003$) in the frequency of

development of a 35 mm follicle, submission rate for live cover/AI, ovulation, and pregnancy compared to Control mares on treatment day 56 (March 29). Interval to first 35 mm follicle was 51.8 ± 4.9, 15.1 ± 4.9, and 23.5 ± 4.9 d (LSmean ± SEM) for Control, GnRH-14 and GnRH-28, respectively. Interval to pregnancy was 65.3 ± 6.8, 27.9 ± 7.1, and 29.2 ± 6.8 d (LSmean ± SEM) for Control, GnRH-14 and GnRH-28, respectively,excluding one GnRH-14 mares that failed become pregnant over four cycles. By the end of the treatment period (Mar. 29), only 21% of Control mares were pregnant compared to 79% of GnRH treated mares. Further, mean serum concentrations of progesterone were similar to (GnRH-28; $P = 0.26$) or greater than (GnRH-14; $P = 0.01$) that of Control mares from day 0 to 46 post-breeding. These data illustrate that continuous administration of native GnRH is a practical and highly efficient option for managing seasonal anovulation in mares.

4.2 Introduction

Approximately 85% of mares transition into an anovulatory state during the early to late fall (September-December) and remain anovulatory for variable periods until after the spring equinox (Ginther, 1992). However, a majority of breed registries utilize the universal birth dates of January 1 and July 1 for foals born in the northern and southern hemispheres, respectively (Ginther, 1992; Davies-Morel, 2003). Thus, foals born as near to those dates as possible within respective locations have a competitive edge during the first 2 yr of competition because of their greater maturity. This has forced breeders to adopt an operational breeding season that begins well before the natural resumption of

ovarian cycles. Currently, the provision of supplemental lighting for approximately 3 mo, beginning before the winter equinox, is the only practical intervention strategy available for advancing the operational breeding season (Ginther, 1992; Davies-Morel, 2003).

As summarized in a recent review (Williams et al., 2012), continuously-applied, native GnRH (as opposed to GnRH agonists) has been shown to provide an efficacious pharmacological strategy for initiating follicular development and ovulation in mares in deep winter anovulation (Johnson, 1987; Becker and Johnson, 1992; Hyland et al., 1987), and for accelerating the timing of pregnancy of mares in late transition (Hyland et al., 1987; Hyland and Jeffcott, 1988). However, this approach has not been tested for its ability to routinely accelerate the timing of pregnancy in anovulatory mares several months before natural reproductive transition. Moreover, there are no commercially-available formulations for delivering native GnRH to achieve this end. Thus, our long-term objectives are to develop managerial principles using native GnRH that successfully addresses these limitations.

In studies reported in an accompanying manuscript, 28-d continuous GnRH treatment, begun in either early February or early March, rapidly increased follicular growth and frequency of ovulation in anovulatory mares. However, mares in which cycles were induced in February (North American) failed to continue to cycle in March. McCue (1992) demonstrated that deeply anestrous mares (February) that were stimulated to develop ovulatory follicles using twice daily injections of buserelin, and induced to ovulate with hCG, exhibited normal luteal function and maintained

pregnancies at a normal rate (< 10% pregnancy loss). Taken together, the foregoing indicate that ovarian cyclicity, CL function, and pregnancy maintenance will be normal if, following development of a large follicle, ovulation is induced with hCG or other gonadotropin surge-inducing methodology. Herein, we tested the hypothesis that continuous native GnRH therapy, in combination with hCG-induced ovulation, would advance the timing of pregnancy by up to 2 mo in winter anovulatory mares beginning in early February (North America).

4.3 Materials and methods

The Institutional Animal Care and Use Committee of Texas A&M University approved in advance all procedures used in this study.

4.3.1 Animals and diets

A total of 42 standard horse mares (Quarter Horse and grade) were utilized at two locations: Animal Reproduction Laboratory, Texas A&M AgriLife Research Station, Beeville (Location 1), and Texas A&M University Horse Center, Texas A&M University, College Station (Location 2). Mares were stratified by age, body condition score (BCS), and size of largest follicle across the two locations in a randomized design. Mares (21 per location) were maintained on pasture and supplemented with a coastal hay and a pelleted concentrate (Crossroad All-Stock CRS Sweet 12) as needed to maintain an average BCS of 5 (1 to 9 scale; Henneke et al., 1983). Mares had continual access to water and trace mineralized salt throughout the study.

4.3.2 Experimental design and treatment allocation

In January, 3 wk before the start of the study, mares were examined once weekly using transrectal ultrasonography (Honda HS-Feb500V, Honda Electronics Co., LTD) to confirm establishment of the winter anovulatory state. In addition, pre-study jugular blood samples were collected once weekly for determination of progesterone concentrations as an adjunct to ultrasonography for assessing ovarian status. Only mares without an ultrasonically-definable CL, serum progesterone ≤ 1 ng/mL, and no follicle > 29 mm during this 3-wk period were used on the study. However, 7 of 42 mares had follicles > 20 mm at the start of the experiment. Mares were stratified by age, BCS, and size of largest follicle and assigned randomly to 1 of 3 groups (n = 14/group): 1) Control; untreated controls, 2) GnRH-14 (GnRH delivered subcutaneously in saline at a rate of 100 µg/h for 56 d with pumps changed at 14-d intervals (Feb. 1 – Mar. 29), or 3) GnRH-28 (GnRH treatment delivered as in 2 for 56 d, with pumps changed at 28 d intervals). Groups 2 and 3 were applied using four 2ML2 (GnRH-14) or two 2ML4 (GnRH-28) Alzet osmotic pumps (Durect Corporation, Cupertino, CA), respectively. Each pump had a volume of 2 ± 0.2 mL, and delivered a solution of GnRH (20 or 40 mg/mL) in saline at a rate of 5.0 (2ML2) or 2.5 µL/h (2ML4) for 14 (GnRH-14) or 28 (GnRH-28) d, respectively. For GnRH-14 and GnRH-28, each consecutive pump was inserted at the end of the functional term of the previous pump. The basis of the GnRH-14 vs GnRH-28 osmotic pump comparison in this study was to ascertain whether functional differences could be detected between those placed subcutaneously over a 28 vs 14-d period in mares maintained in a natural outdoor environment.

4.3.3 Osmotic pump insertion

Mares were placed in a stock and sedated with detomidine HCl (Dormosedan, Pfizer Animal Health, New York, NY) at a rate of 20 to 40 µg/kg BW, if required. An area at the base of the neck (anterior to the scapula) was clipped, scrubbed with an iodophore, and disinfected with povidone iodine solution and 70% isopropanol. Three to five milliliters of 2% lidocaine HCl (Agri Laboratories, St. Joseph, MO) was injected subcutaneously and a 2- to 3-cm incision made through the skin with a sterile scalpel. A sterile, blunt instrument was used to expand the subcutaneous space making a pocket to accommodate the GnRH pump. The incision was closed with non-absorbable synthetic suture that was left in place for 7 d and an equine approved dressing applied to the site of incision. After 14 or 28 d, spent GnRH pumps were removed and replaced with a functional GnRH pump at another location at the base of the neck on the contralateral side as described above. Evacuated subcutaneous pockets were flushed with dilute povidone iodine solution followed by surgical closure with non-absorbable synthetic suture. All GnRH pumps were surgically removed at the end of the study. Mares observed with inflamed or infected skin were cleaned daily, disinfected with povidone iodine, and an antibacterial ointment was applied. This occurred only rarely.

4.3.4 Teasing score

Mares were teased daily in a large pen of 5 to 6 mares using an intact stallion in an adjacent pen separated by a solid panel. Teasing scores were assessed on a scale of 1 to 4 (1, mare breaks down in the presence of the stallion; 2, vulvar winking in the presence of the stallion; 3, indifference to the stallion's presence; 4, rejects stallion). Only mares with a teasing score of 1 were bred.

4.3.5 Ultrasonography

Transrectal ultrasonography (Honda HS-Feb500V, Honda Electronics Co., LTD; Aloka SSD-900V, Aloka, Inc.) was performed every 3 d until a follicle measuring ≥ 30 mm was detected. Once a follicle measuring 30 mm was observed, ultrasonography was performed daily to confirm time of ovulation following natural service or AI and treatment with hCG (see description below). Beginning 14 d after the day of confirmed ovulation, mares were examined for the presence of an embryonic vesicle. Detected pregnancies were confirmed on days 24, 33, and 45 on the basis of embryonic development and presence of a fetal heartbeat. Mares found to be non-pregnant were returned to the previous ultrasonography schedule for non-pregnant mares until confirmation of pregnancy or through 4 estrous cycles if not pregnant.

4.3.6 Natural service and artificial insemination

When a follicle ≥ 35mm was first observed ultrasonographically and estrus detected, mares were either bred on the following day (~ 24 h) by natural service using 1

of 2 fertile, experienced stallions (Location 1) or were bred by AI using extended cooled semen collected from 1 of 2 stallions with known fertility (Location 2). Immediately following natural service/AI, each mare received 2000 IU hCG (2 mL im, Chorulon; Intervet Inc., Millsboro, DE) to induce ovulation. Stallions used at each location for natural service or semen collection underwent a standard breeding soundness examination. The majority (> 90%) of mares at each location were bred/inseminated using a single predominant stallion at each location. A few mares at each location were booked to other stallions and were bred/inseminated using the alternate stallions. Artificial insemination breeding doses contained at least 500 million progressively motile sperm cells in a standard extender (E-Z Mixin CST, Animal Reproduction Systems, Chino, CA). Dismount samples were collected for each live cover breeding for assessment of sperm cell number and motility. Natural service and artificial insemination were continued through June.

4.3.7 Blood collection

Single blood samples (10 mL) were collected via jugular venipuncture into sterile blood collection tubes with no additives (Tyco Healthcare Group LP, Mansfield, MA). Samples were collected once weekly during the pre-treatment period, then every other day beginning on the first day of breeding/insemination until 46 d post-breeding for determination of serum concentrations of progesterone. Samples were placed on ice or under refrigeration (4° C) for approximately 1 h and then allowed to clot at room temperature for approximately 45 min prior to centrifugation. Samples were centrifuged

for 45 min at 4°C, and serum harvested and stored at -20˚C until determination of progesterone by RIA.

4.3.8 Hormone analyses

Serum concentrations of progesterone were determined using a commercial RIA kit assay (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA) as reported previously from this laboratory (Williams et al., 2007). The interassay CV of the low and high references averaged 3.7 and 4.2%, while the intraassay CV was 7.4%.

4.3.9 Statistical analysis

Frequency data (presence of a follicle ≥ 35 mm, breeding, ovulation, and pregnancy) were analyzed using the CATMOD procedure of the Statistical Analysis System (SAS Inst. Inc., Cary, NC) in order to account for location effects in the model and the Fisher's Exact Test of PROC FREQ. Analysis of repeated measures was used to determine main effects of treatment on serum concentrations of progesterone utilizing the PROC MIXED procedure of SAS. Fixed effects were treatment, location, day, and treatment x day interaction. The variable 'day' served as the repeated variable and 'mare' as the subject. The PROC GLM procedure of SAS was used to determine main effects of treatment on reproductive event intervals. Sources of variation were treatment, location, and treatment by location interaction. The LS Means statement was used to compare means when significant differences were detected using PROC MIXED and PROC GLM, respectively. Pearson correlation coefficients were determined using

PROC CORR, while linear regression analysis was performed using PROC REG to relate initial follicle size to interval to first breeding. Main effects were considered significant when $P \leq 0.05$ and a trend toward significance when $P \leq 0.10$.

4.4 Results

4.4.1 Reproductive end-points

Location had no effect ($P \geq 0.48$) on any of the reproductive end-points assessed. No differences ($P \geq 0.48$) were observed between GnRH-14 and GnRH-28; thus, the treatment groups were pooled for comparisons of reproductive end-points to controls. Mares treated with GnRH had a markedly increased frequency of development of a 35 mm follicle ($P = 0.003$), submission rate for live cover/AI ($P = 0.003$), ovulation ($P = 0.0002$), and pregnancy ($P = 0.0004$; Fig. 4.1) compared to Control mares by the end of the treatment period (March 29). By the end of the treatment period (March 29), only 21.4% of Control mares were pregnant compared to 78.6% of GnRH treated mares. Mean intervals to each reproductive end-point are summarized in Table 4.1 and demonstrate clearly the large effects of continuous GnRH treatment on reproductive variables compared to controls.

Fig. 4.1. Cumulative incidence of reproductive events of first: 35 mm follicle, breeding, ovulation, and pregnancy for GnRH therapeutics trial during the 56 day treatment period. Treatments were control: untreated (n = 14); GnRH-14: GnRH delivered subcutaneously in saline at a rate of 100 μg/h for 8 wk (Feb. 1 – Mar. 29) using four consecutive 14-d pumps (Alzet 2ML2; n = 14), and GnRH-28: GnRH delivered as in GnRH-14, but using two, 28-d pumps (Alzet 2ML4; n = 14). GnRH treatments did not differ ($P \geq 0.48$) and therefore data are pooled in this figure (n = 28). Asterisks denote significant differences ($P \leq 0.03$) between treatments within reproductive events.

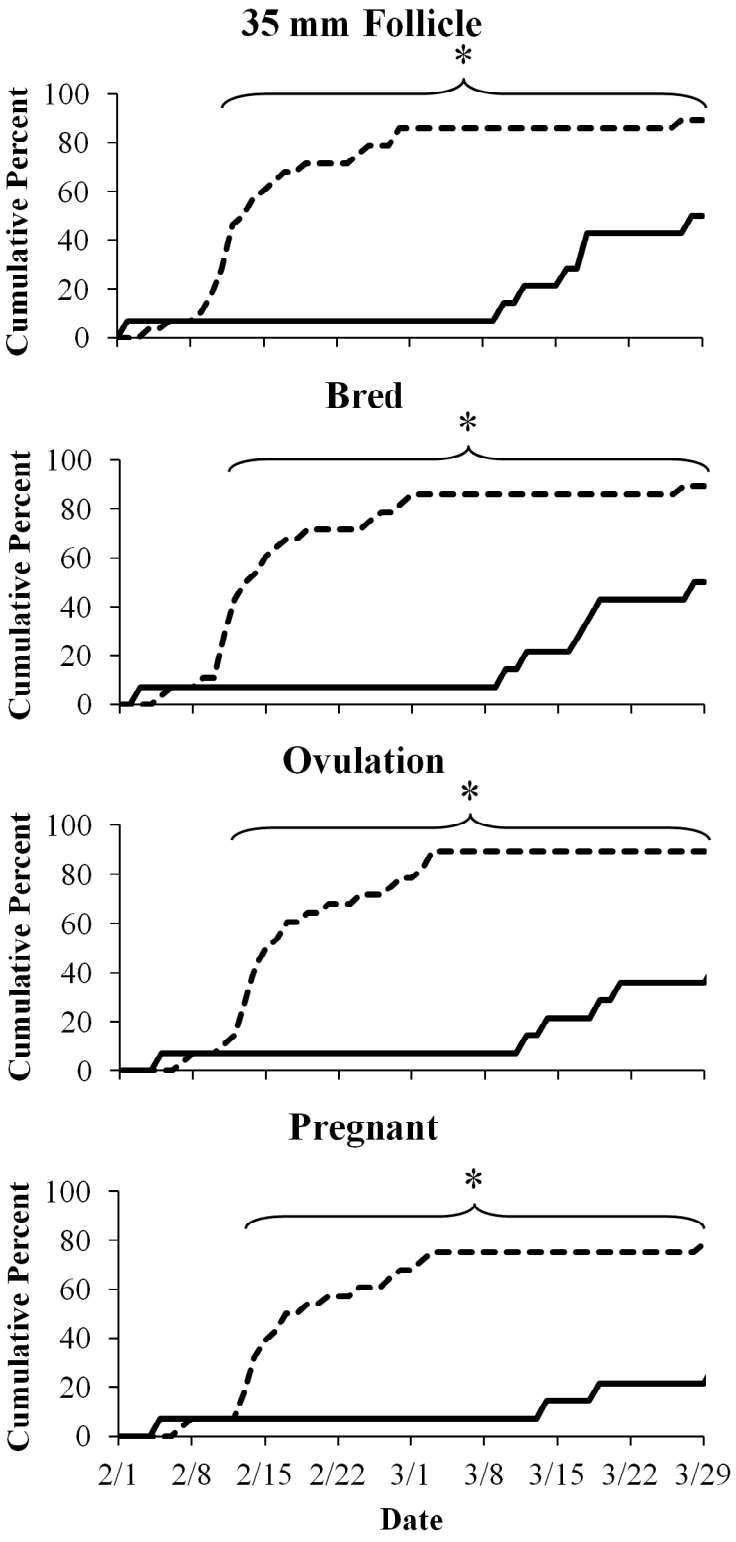
35 mm Follicle
Bred
Ovulation
Pregnant
Cumulative Percent
100
80
60
40
20
0
*
2/1
2/8
2/15
2/22
3/1
3/8
3/15
3/22
3/29
Date

Table 4.1. Least squares means (± SEM) interval to first 35 mm follicle, breeding, ovulation, and pregnancy for mares in GnRH therapeutics trial. Mare treatments were control: untreated (n = 14), GnRH-14: GnRH delivered subcutaneously in saline at a rate of 100 µg/h for 8 wk (Feb. 1 – Mar. 29) using four consecutive 14-d pumps (Alzet 2ML2; n = 14, n = 14, n = 14, and n = 13 for 35 mm follicle, ovulation, breeding, and pregnancy, respectively), and GnRH-28: GnRH delivered as in GnRH-14, but using two, 28-d pumps (Alzet 2ML4; n = 14). [a,b,c]Means without a common superscript letter differ ($P \leq .0006$) within reproductive events.

	Treatment		
Intervals to first, days:	**Control**	**GnRH-14**	**GnRH-28**
35 mm follicle	51.8 ± 4.9[a]	15.1 ± 4.9[b]	23.5 ± 4.9[b]
Breeding	52.1 ± 4.9[a]	15.5 ± 4.9[b]	23.8 ± 4.9[b]
Ovulation	55.6 ± 4.9[a]	17.4 ± 4.9[b]	26.2 ± 4.9[b]
Pregnancy	65.3 ± 6.8[a]	27.9 ± 7.1[b]	29.2 ± 6.8[b]

4.4.2 Serum concentrations of progesterone

Location tended to influence serum concentrations of progesterone with mares in College Station (11.0 ± 1.2 ng/mL) tending to have increased ($P \geq 0.09$) serum concentrations of progesterone compared to mares in Beeville (8.0 ±1.3 ng/mL). No treatment by day interaction ($P = 0.17$) was observed for serum concentrations of progesterone during induced (GnRH-treated) and naturally-occurring (Control) luteal phases; however, mean serum concentrations of progesterone tended to be greater ($P = 0.06$) in GnRH-14 (12.7 ± 1.6 ng/mL) than GnRH-28 (9.3 ± 1.6 ng/mL) and greater ($P = 0.0006$) in GnRH-14 than in Control mares (6.7 ± 1.5 ng/mL) that ovulated during the entire experiment. As expected, serum concentrations of progesterone increased ($P < 0.0001$) as pregnancy progressed (Fig. 4.2).

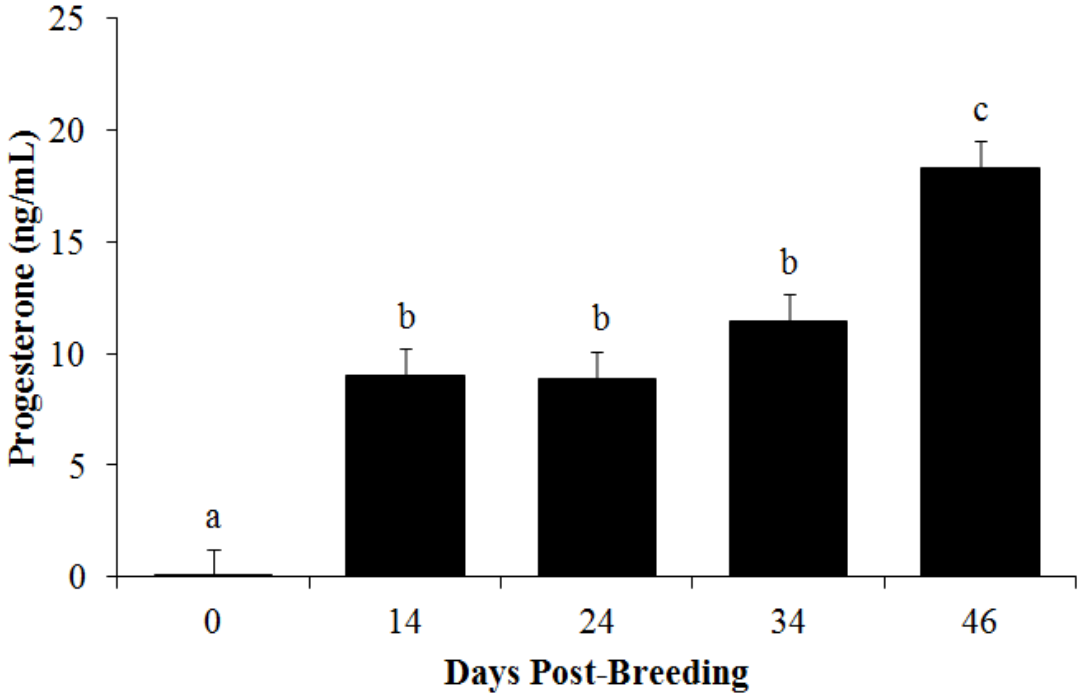

Fig. 4.2. Serum concentration of progesterone (LS Means + SEM) from day 0 to 46 post-breeding for mares in GnRH therapeutics trial. Mare treatments were control: untreated (n = 14, 14, 14, 14, and 13 for days 0, 14, 24, 34, and 46, respectively), GnRH-14: GnRH delivered subcutaneously in saline at a rate of 100 µg/h for 8 wk (Feb. 1 – Mar. 29) using four consecutive 14-d pumps (Alzet 2ML2; n = 14, 14, 12, 12, and 12 for days 0, 14, 24, 34, and 46, respectively), and GnRH-28: GnRH delivered as in GnRH-14, but using two, 28-d pumps (Alzet 2ML4; n = 14, 13, 13, 13, and 13 for days 0, 14, 24, 34, and 46, respectively). The interaction of treatment by sample was not significant (P = 0.17) and therefore only sample (P < .0001) effects are represented. [a,b,c]Means without a common superscript letter differ ($P \leq 0.0001$) between samples.

4.4.3 Pregnancy status

Of the 22 GnRH-treated mares that became pregnant during the 56-d experiment, 19 maintained pregnancy through day 45. The three mares exhibiting embryonic loss were GnRH-28 mares and embryos began to exhibit ultrasonographic changes indicative of pending death on days 24, 33 and 45. None of these dates were associated with declines in serum concentrations of progesterone or GnRH treatment removal. Two of

the three mares re-ovulated within 9 d following loss of pregnancy and maintained subsequent pregnancies through the final day of confirmation (day 45). The third mare remained anovulatory through her last ultrasound exam on April 21 as a result of formation of secondary corpora lutea during the initial pregnancy and not due to a return to the winter anovulatory state. By the end of the breeding season (June 30th), 92% (24 of 26) of GnRH-treated and 86% (12 of 14) of Control mares were at least 45 days pregnant. Additionally, all mares were allowed to go to term, with all mares foaling normally and no abnormalities observed.

4.4.4 Follicular responsiveness

A small proportion of GnRH-treated (3 of 28) and Control mares (4 of 14) had initial follicle diameters before the start of the experiment greater than 20 mm in diameter. However, interval to first breeding was not reduced ($P = 0.15$) in mares with initial follicle size > 20 mm (27.7 ± 7.1) compared to mares with initial follicle size ≤ 20 mm (39.6 ± 3.5; Figure 4.3). No differences ($P = 0.90$) were observed between GnRH-14 and GnRH-28; thus, the treatment groups were pooled for further comparisons to controls. The relationship between initial follicle size and interval to first breeding was not significant ($P = 0.15$; $y = -1.918x + 88.37$; $R^2 = 0.17$) in Control mares but tended to be significant ($P = 0.09$; $y = -1.3097x + 40.924$; $R^2 = 0.11$) in GnRH treated mares (Fig. 4.3). Multiple ovulations were not evident in any of the mares involved in this study.

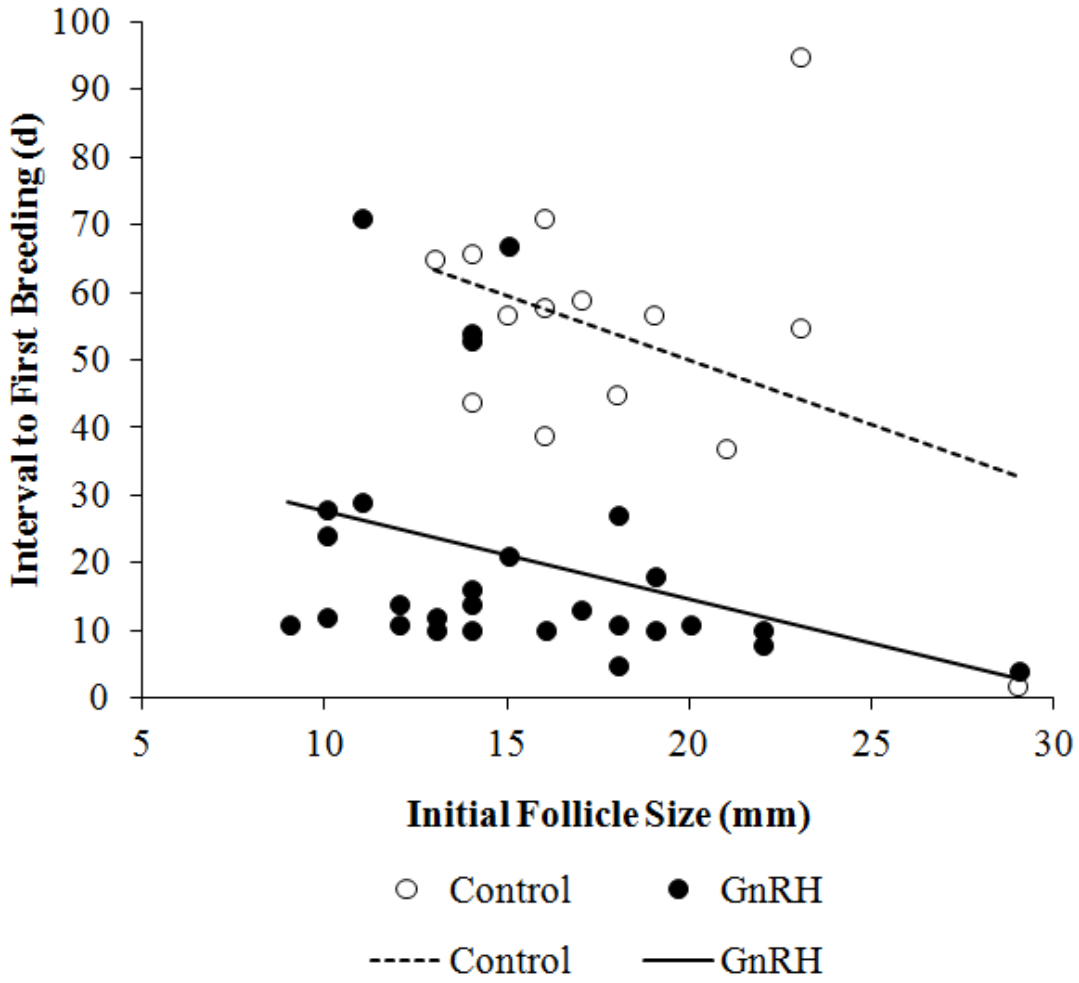

Fig. 4.3. Correlation between initial follicle size (mm; x-axis) and interval to first breeding (days; y-axis) for mares in GnRH therapeutics trial. Mare treatments were control: untreated (n = 14; y = -1.918x + 88.37; R^2 = 0.17; *P* = 0.15), GnRH-14: GnRH delivered subcutaneously in saline at a rate of 100 µg/h for 8 wk (Feb. 1 – Mar. 29) using four consecutive 14-d pumps (Alzet 2ML2; n = 14), and GnRH-28: GnRH delivered as in GnRH-14, but using two, 28-d pumps (Alzet 2ML4). GnRH treatments did not differ (*P* = 0.90) and therefore data are pooled in this figure (n = 28; y = -1.3097x + 40.924; R^2 = 0.11; *P* = 0.09).

4.5 Discussion

Results of the current study illustrate that continuous, subcutaneous infusion of native GnRH (100 µg/h) is a highly-effective approach for accelerating onset of reproductive transition, ovulation, and pregnancy in mares in deep winter anovulation. The marked increase in pregnancy rate was realized using both AI and natural service under standard breeding farm conditions typical of those found in North America and globally. Using this approach, once a large follicle (35 mm) was attained using continuous GnRH infusion, mares were bred and an ovulation-inducing dose of hCG was administered at the time of breeding.

Although a number of previous reports have demonstrated the ability of continuously-infused, native GnRH to initiate follicular development and ovulation in mares in deep winter anestrus (Johnson, 1987; Becker and Johnson, 1992), breeding studies conducted in New Zealand focused on mares in late reproductive transition/shallow anestrus (Hyland et al., 1987; Hyland and Jeffcott, 1988). Therefore, to our knowledge, the work reported herein is the first demonstrating the ability to successfully incorporate such a strategy through successful pregnancy in mares in deep winter anestrus. Also, using GnRH agonist injected twice daily, other investigators have induced follicular development and ovulation, and accelerated the timing of pregnancy in winter anestrous mares (McCue et al., 1992); however, when GnRH agonist was administered in a practical manner (continuously) the treatments were not effective because of apparent pituitary desensitization (Bergfelt and Ginther, 1992; Fitzgerald et al., 1993; Mumford et al., 1994). Hence, the value of the current work is the unequivocal

demonstration that the continuous, subcutaneous infusion of the native hormone, when began well in advance of the onset of vernal transition, is a highly efficacious approach for accelerating the breeding season by 30 to 60 days.

An important aspect associated with GnRH treatments that accelerate pregnancy in the winter anovulatory mare is that related to corpus luteum function and the maintenance of pregnancy. As shown in an accompanying manuscript (Thorson et al., 2010), if mares are treated continuously with GnRH for 28 d, beginning in early February, a rapid decline in circulating LH and resumption of winter anovulation occurs at treatment withdrawal in non-bred mares. To the contrary, if mares are treated for 28 d beginning in early March, ovarian cycles continue unabated following cessation of treatment, as mares are approaching the natural breeding season at this time. These findings pose the concern that subnormal luteal function and pregnancy loss could potentially occur if mares in February or earlier are bred and conceive during GnRH-induced cycles in which ovulation is allowed to occur spontaneously. Hyland and Jeffcott (Hyland and Jeffcott, 1988) addressed this issue to some extent in a small number of mares treated continuously with GnRH during late transitional anestrus. Pregnancy was maintained through at least day 45 of gestation in 4 of 5 GnRH-treated mares. However, when mares in deep winter anestrus were treated with twice daily GnRH agonist injections (McCue et al, 1992 Bergfelt and Ginther, 1992), different results were observed, depending upon time of treatment onset and management of the preovulatory follicle. In the work by Bergfelt and Ginther (1992), mares treated with twice daily injections of deslorelin in February became pregnant (64%); however, this

population exhibited a very high rate of embryonic loss (64%). Yet, mares treated similarly in April responded well (71% pregnancy rate) and embryonic losses were reduced (20%). However, McCue et al (1992), using twice daily injections of another GnRH agonist (buserelin) in deep winter anestrous mares, reported high pregnancy rates (> 70%) and only a 7% embryonic loss. In that study, when a preovulatory follicle was detected in response to GnRH agonist treatment, mares were induced to ovulate using hCG. In the current study using continuous infusion of native GnRH, ovulation of the preovulatory follicle was also induced with hCG. In our experiment, only 3 of 25 GnRH-treated, pregnant mares failed to maintain pregnancies to term. Collectively, we interpret these findings to indicate that mares responding to continuous GnRH treatments early in the calendar year (February or earlier) are at risk for developing subnormal luteal function and pregnancy loss unless the preovulatory follicle is induced to ovulate using hCG or an ovulatory dose of a commercially-available GnRH agonist (e.g., Sucramate). The latter ensures an adequate gonadotropic environment to support normal CL formation. Although the question needs further clarification, current evidence indicates that the amount of circulating LH needed to develop a preovulatory follicle and spontaneous ovulation/luteinization may be much less than that needed for optimal luteal formation and function.

In the current study, there was no indication of subnormal luteal function in GnRH treated mares. In fact, mean serum concentrations of progesterone were similar (GnRH-28) or significantly greater (GnRH-14) during the luteal phases of GnRH-treated mares than in the control mares, and were typical of those observed during the natural

breeding season (Ginther, 1992; Nett et al., 1976). Bergfelt and Ginther (1992) reported that mares with minimal ovarian activity at initiation of GnRH treatment where more likely to develop dysfunctional CL but in that study mares were allowed to ovulate spontaneously. Thus, as noted earlier, the gonadotropic environment immediately preceding ovulation may play a key role in CL competence within ovarian cycles outside of the natural breeding season. Gonadotropic requirements of the equine CL have not been studied as extensively as in other species. However, in other female mammals, adequate LH support during ovulation and early development of the CL appears to be a key requirement (Astwood, 1941; Spies et al., 1967; Vande Wiele et al., 1970), whereas the amount of circulating LH thereafter is of lesser relevance (Snook et al., 1969; Juengel et al., 1995). Although 3 GnRH-treated mares experienced embryonic loss by day 45 of pregnancy in the current study, there was no measurable decline in serum concentrations of progesterone until after death of the embryos. Deficiencies in serum progesterone concentrations during pregnancy, defined as < 2.5 ng/mL (Douglas et al., 1985), was not observed in the current experiment but was a notable feature of pregnancy loss in a previous study using a GnRH agonist (Bergfelt and Ginther, 1992).

Responsiveness of mares to exogenous native GnRH treatment in the present study was not enhanced in mares with initial follicle diameter greater than 20 mm compared to mares with initial follicle diameter less than 20 mm, which is similar to our previous observations (Thorson et al., 2010). Other earlier reports have indicated that size of the largest follicle at treatment onset has a major influence on timing and frequency of ovarian responses (Bergfelt and Ginther, 1992; Ginther and Bergfelt,

1990). Perhaps the tendency for mares with larger follicles at the start of treatment to respond more rapidly than small follicles was related to the concentrations of LH achieved with particular treatments.

Multiple ovulations were not observed in GnRH treated mares in the current work as reported by others during the winter anovulatory season (McCue et al., 1992; Ginther and Bergfelt, 1990), spring transition (Raz et al., 2009), and breeding season (Fitzgerald et al., 1993) using potent GnRH agonists. Native GnRH has also been shown to induce multiple ovulations during the anovulatory season. However, this was observed when treatments were delivered intravenously in either a continuous or pulsatile manner (Becker and Johnson, 1992; Johnson and Becker, 1988) but not when delivered subcutaneously (Thorson et al., 2010; Velez et al., 2008). Another factor that may play a role is breed-type of mares involved. Since Quarter Horse mares have a lower natural incidence of multiple ovulations compared to both Thoroughbreds and Standardbreds, it is possible that breed or breed type may contribute to the variability observed for this response (Ginther et al., 1982). Also, Ginther and Bergfelt (1990) reported a greater frequency of multiple ovulations (64%) with the presence of a follicle greater than 25 mm at treatment initiation, while Raz et al. (2009) assigned mares to treatments only after development of a follicle greater than 25 mm and reported a 7% multiple ovulation rate.

In conclusion, continuous treatment of winter anovulatory mares with native GnRH at a rate of 100 μg/h beginning in early-February is an effective means to accelerate the Julian day of pregnancy and support primary and secondary corpora luteal

development and function to maintain pregnancy following treatment withdrawal. Further, interval to first 35 mm follicle, breeding, ovulation, and pregnancy was similar within the two groups of GnRH-treated mares (2ML2 vs 2ML4 pumps). However, because of the need for minor surgery to apply these pumps, it is highly unlikely that such devices will be employed routinely in the breeding industry. Thus, development of a user-friendly and commercially-available platform (e.g., depot technology) to strategically deliver native GnRH in a continuous manner remains in development.

CHAPTER V

HYPOTHALAMIC DISTRIBUTION, ADENOHYPOPHYSEAL RECEPTOR EXPRESSION, AND LIGAND FUNCTIONALITY OF RF-AMIDE RELATED PEPTIDE 3 IN THE MARE DURING THE BREEDING AND NON-BREEDING SEASONS

5.1 Synopsis

RF-amide related peptide 3 (RFRP3), the mammalian homologue of avian gonadotropin-inhibitory hormone (GnIH), has been shown to negatively regulate the secretion of LH and may contribute to reproductive seasonality in some mammalian species. Herein, we examined the presence and potential role of the RFRP3-signaling system in regulating LH secretion in the mare during the breeding and non-breeding seasons. Hypothalamic *RFRP3* mRNA was detected at the level of the dorsomedial nucleus (DMH) and paraventricular nucleus (PVN), but expression did not change with season. A greater number of *RFRP3*-expressing cells were observed throughout the rostral-caudal extension of the DMH. Further, adenohypophyseal expression of the RFRP3 receptor (*GPR147*) during the winter anovulatory season did not differ from that during either the follicular or luteal phases of the estrous cycle. When tested *in vitro* using adenohyphyseal cell culture, or *in vivo* during both the breeding and non-breeding seasons, neither equine nor ovine sequences for RFRP3 suppressed basal or GnRH-mediated release of LH. However, infusion of RF9, an RFRP3 receptor-signaling antagonist, into seasonally-anovulatory mares induced a robust increase in secretion of

LH both before and following continuous treatment with GnRH. Collectively, these results indicate that the cellular machinery associated with RFRP3 function is present in the equine hypothalamus and adenohypophysis. However, evidence for functionality of the RFRP3 signaling network was only obvious when an antagonist of receptor signaling was employed. As GnRH responsiveness was not affected by RF9, results suggest influence at the GnRH neuronal level to stimulate or disinhibit secretion of GnRH.

5.2 Introduction

Reproductive seasonality in the equine species has been studied for several decades. Transition from the breeding to non-breeding season is characterized by a marked decline in synthesis and release of anterior pituitary LH, and the cessation of ovulatory cycles. However, photoperiod-driven signaling pathways in the hypothalamic-hypophyseal axis that underlie this process have not been fully elucidated (Williams et al., 2012).

Limited studies by Alexander and Irvine (1986), utilizing the intercavernous sinus cannulation (ICS) technique, and others using hypothalamic push-pull perfusion (Sharp and Grubaugh, 1987), have indicated that the secretion of GnRH is decreased during the winter anovulatory period, similar to that observed during the non-breeding seasons of other seasonal breeders (Barrell et al., 1992). Moreover, when seasonally anovulatory mares are infused hourly (Johnson, 1987; Becker and Johnson, 1992) or continuously (Becker and Johnson, 1992; Hyland and Jeffcott, 1988; Collins et al., 2007) with native GnRH, anterior pituitary secretion of LH is increased, follicular development

follows, and spontaneous or induced ovulations occur at a high frequency. Thus, regulation of the secretion or action of GnRH appears to be one of the most important factors regulating seasonality in this species.

A potential role for inhibitory neuropeptides in this process has also been proposed (Smith et al., 2012). These would act theoretically by impairing GnRH neuronal activity or through direct suppression of secretion of LH by the gonadotrope. Tsutsui et al. (2000) was the first to report on the existence of a novel hypothalamic neuropeptide with inhibitory effects on secretion of LH in quail. The molecule contained a C-terminal LPLRF-amide sequence and was coined gonadotropin-inhibitory hormone (GnIH). Three RF-amide related peptides (RFRP1, 2 and 3) and two G-protein coupled receptors (GPR147/NPFFR1 and GPR74/NPFFR2) have been characterized in mammalian species. In rats, hamsters, sheep, and cattle, RFRP3 has been shown to suppress the secretion of LH (Kriegsfeld et al., 2006; Clarke et al., 2008; Kadokawa et al., 2009; Pineda et al., 2010) as well as adenohypophyseal responsiveness to GnRH (Clarke et al., 2008; Kadokawa et al., 2009; Pineda et al., 2010) in a dose-dependent manner. Thus, if RFRP3 regulates seasonality in mammals, it may do so in part by direct effects on the adenohypophysis. However, its role in mammalian reproduction continues to remain controversial, as the number of reports showing functional effects (Kriegsfeld et al., 2006; Clarke et al., 2008; Pineda et al., 2010) has been offset by an equally significant number showing little or no effect in rats, hamsters and sheep (Murakami et al., 2008; Anderson et al., 2009; Rizwan et al., 2009; Ancel et al., 2012; Caraty et al., 2012; Ubuka et al., 2012).

The existence and function of RFRP3 in the mare has not been reported. Experiments reported herein addressed the hypotheses that expression of genes for RFRP3 and its receptor occurs within the equine hypothalamus and adenohypophysis, respectively, and that the predicted sequence for equine RFRP3 (eRFRP3) functionally regulates secretion of LH in the mare.

5.3 Materials and methods

All animal-related experiments were approved by the Institutional Agricultural Animal Care and Use Committee (IAACUC) of the Texas A&M University System.

5.3.1 RFRP3 sequence, synthesis and application

Using primers designed for the *eRFRP* gene, a partial cDNA sequence of approximately 420 bp was amplified by PCR and cloned into a vector. Based on cDNA sequencing, the predicted sequence of the eRFRP3 has been determined to be Ile-Pro-Asn-Leu-Pro-Gln-Arg-Phe-NH2. The peptide synthesized for studies reported herein have been validated by high-performance liquid chromatography and mass spectral analysis (98% purity and molecular weight of 983). Doses of eRFRP3 used in the following experiments were determined by doses used previously in other species such as sheep (Clarke et al., 2008) and rats (Murakami et al., 2008).

5.3.2 Experiment 1. Seasonal expression of the eRFRP gene

5.3.2.1 Animal model and experimental design

Twelve mares with histories of regular reproductive cycles were used to determine whether season influences the expression of eRFRP. Circulating concentrations of progesterone and transrectal ultrasonography were used to confirm reproductive status before the beginning of the experiment. Four mares were euthanized during the winter anovulatory season (December-January) once anovulatory status was confirmed via serum progesterone and ultrasonography. The remaining eight mares were euthanized during the follicular (n = 4) and luteal (n = 4) phases of the estrous cycle within the natural breeding season (May-July). After euthanasia, a tissue block containing the hypothalamus was dissected out, rapidly frozen in liquid nitrogen vapor, and stored at -80^{o} C. Coronal sections (20 μm) were cut on a cryostat and a series of every 10th section was used for detection of eRFRP3 precursor mRNA by isotopic *in situ* hybridization as previously described (Redmond, 2010). A ^{35}S-labeled riboprobe was synthesized using the *equus caballus* RFRP cDNA and processed as described previously (Mitchell and Ing, 2003) using promoter specific primers. Controls included hybridization with sense probe. The location of *eRFRP* mRNA-containing neurons were identified under dark- and bright-field microscopy based on density of silver grains 5x above background density. Cell counts were performed in comparable sections within the paraventricular nucleus (PVN; 5 sections) and the dorsomedial nucleus of the hypothalamus (DMH; 10 sections) of each mare.

5.3.2.2 Hormone assays

Serum concentrations of progesterone were determined using a commercial RIA kit (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA) as reported previously from this laboratory (Williams et al., 2007). All samples were analyzed for progesterone in a single assay with minimum detectable concentration of 0.1 ng/mL and a mean intraassay CV of 6.3%.

5.3.2.3 Statistical analysis

The main effect of reproductive status on number of cells containing *eRFRP3* mRNA were compared between the groups using the Proc GLM procedure of the Statistical Analysis System (SAS; SAS Inst. Inc., Cary, NC). Source of variation was reproductive status. The Least Squares Means procedure was used to compare means if significant differences were detected.

5.3.3 Experiment 2. Adenohypophyseal expression of eGPR147 (RFRP3 receptor) during the breeding and non-breeding seasons

5.3.3.1 Animal model and experimental design

Adenohypophyses of mares used for Experiment 1 were used. Adenohypophyses were snap frozen in liquid nitrogen and stored at -80^{o}C. Cellular RNA was isolated from adenohypophyseal tissue and reverse transcribed (RT) to cDNA as described previously (Allen et al., 2012). Initially, we performed PCR reactions to amplify equine GPR147 (eGPR147) from adenohypophyseal tissue using primers that successfully amplified

GPR147 from the hypothalamus. Following successful PCR amplifications, real-time RT-PCR was performed to quantify *eGPR147* gene expression in the adenohypophysis of mares (Experiment 1) during the winter anovulatory season (n = 4; December-January) and during the follicular (n = 4) and luteal (n = 4) phases of the estrous cycle within the natural breeding season (May-July). Primers used in the real-time RT-PCR analysis were designed based on the sequence of eGPR147 amplified from equine hypothalamus. Reactions were performed in triplicate for each cDNA sample as described previously (Allen et al., 2012). Controls include the verification of absence of primer-dimer amplification and genomic DNA carryover, and estimation of melting temperature of PCR products.

5.3.3.2 Statistical analysis

Real-time RT-PCR data were analyzed after normalizing mean gene expression to a reference gene (*RPS20*). The main effects of reproductive status on *GPR147* gene expression were compared between the groups using the Proc GLM procedure of SAS. Source of variation was reproductive status. The Least Squares Means procedure was used to compare means if significant differences were detected.

5.3.4 Experiment 3. Effects of eRFRP3 on GnRH-mediated secretion of LH from adenohypophyseal cell culture

5.3.4.1 Animal model and experimental design

Adenohypophyses were harvested from five mares after euthanasia and placed directly on ice. Since pituitaries were collected during the winter, all mares received Alzet osmotic pumps delivering GnRH (100 μg/h) for 12-18 d prior to euthanasia to stimulate synthesis of LH in gonadotropes. Adenohypophyseal cells were dispersed enzymatically, plated in 6-well plates containing 300,000 cells/well, and cultured in Dulbecco's modified Eagle's medium following procedures described previously (Welsh et al., 1986). On day 4 (after cells reach approximately 70% confluence), cells were incubated with media alone (control), media containing GnRH (10^{-8} M) only (positive control), or media containing GnRH (10^{-8} M) plus eRFRP3 (10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} M) for 4 h. Each treatment was applied to at least 3 wells and 5 independent replications were performed. Additional cells were also treated with GnRH (10^{-8} M) plus ovine RFRP3 (oRFRP3; 10^{-6} and 10^{-9} M) for 4 h to test a preparation that has been shown previously to inhibit release from ovine gonadotropes (Clarke et al., 2008) as well as a preparation that has been shown previously to have no effect in ovine gonadotropes (Dr. Terry Nett, Colorado State University, personal communication). At the end of each 4-h incubation period, culture media was harvested and stored at -20^{o}C.

5.3.4.2 Hormone analyses

Concentrations of LH in media were analyzed by double antibody RIA, as validated previously in this laboratory (Williams et al., 2007). A highly-purified equine LH (eLH AFP-5130A) preparation was used for both iodinated tracer and standards. An anti-eLH antiserum (AFP-240580) was used at a dilution of 1:120,000 which yielded 28.3% binding on average at B/O. The sensitivity of the LH assays were 0.1 ng/mL and mean intra- and interassay CV were 7.2% and 3.7%, respectively.

5.3.4.3 Statistical analysis

The Proc GLM procedure of SAS was used to determine main effects of RFRP3 treatment on differences in media LH concentrations. Sources of variation were treatment, replicate, and treatment x replicate interaction. The Least Squares Means procedure was used to compare means if significant differences were detected.

5.3.5 Experiment 4. Stimulatory effects of an RFRP3 receptor-signaling antagonist (RF9) on secretion of LH during the winter anovulatory period

5.3.5.1 Animal model and experimental design

Ten winter anovulatory mares were assigned randomly to one of two treatments: Control or RF9 (n = 5/treatment). RF9 (Adamantanecarbonyl-Arg-Phe-NH4), a selective antagonist for the NPFF receptors, was synthesized by GeneCust Europe (Dudelange, Luxembourg). On experimental day 0, jugular blood samples were collected at 10 min intervals for 5 h beginning 1 h before treatment. Intravenous injections of saline or RF9

(0.2 mg/kg BW) were administered at hour 1, 2, and 3. Mares were then treated continuously with GnRH (100 µg/h) for 72 h via subcutaneous osmotic pumps, an approach uniquely effective in the equine for stimulating adenohypophyseal synthesis of LH. On experimental day 4 (12 h after pump removal), jugular blood samples were collected at 10 min intervals for 6 h beginning 1 h before treatment. Intravenous injections of saline or RF9 (0.4 mg/kg BW) were administered at 1 and 2 h. A GnRH injection (250 µg, intravenously) at hour four was conducted to test adenohypophyseal responsiveness. At the end of the sampling periods, plasma was harvested and stored at -20^{o}C until determination of plasma LH concentrations using a previously validated equine LH RIA (Williams et al., 2007).

5.3.5.2 Hormone analyses

Radioimmunoassay for LH was the same as for previous experiments. Mean intra-assay CV was 7.4%, with all samples analyzed in one assay.

5.3.5.3 Statistical analysis

The GLM procedure of SAS was used to determine main effects of RF9 treatment on differences in plasma concentrations of LH. Sources of variation were treatment, period, and treatment x period interaction. The Least Squares Means procedure was used to compare means when significant differences were detected. Area

under the curve was calculated using the Trapezoid Rule. The Proc T-test procedure of SAS was used to determine main effects of GnRH-injection on differences in plasma LH concentrations.

5.4 Results

5.4.1 Experiment 1. Seasonal expression of the eRFRP gene

Hypothalamic *RFRP* mRNA was detected within the DMH and PVN (Fig. 5.1). In the PVN, *RFRP*-expressing cells were distributed sparsely. A greater number of *RFRP*-expressing cells was observed throughout the rostral-caudal extension of the DMH. However, *RFRP*-expressing cell count within the PVN and DMH during the winter anovulatory season (PVN, 53.5 ± 20.7; DMH, 314.3 ± 59.0) did not differ ($P \geq 0.44$) from that during either the follicular (PVN, 39.0 ± 16.1; DMH, 248.5 ± 89.1) or luteal (PVN, 46.3 ± 32.2; DMH, 172.5 ± 72.2) phases of the estrous cycle.

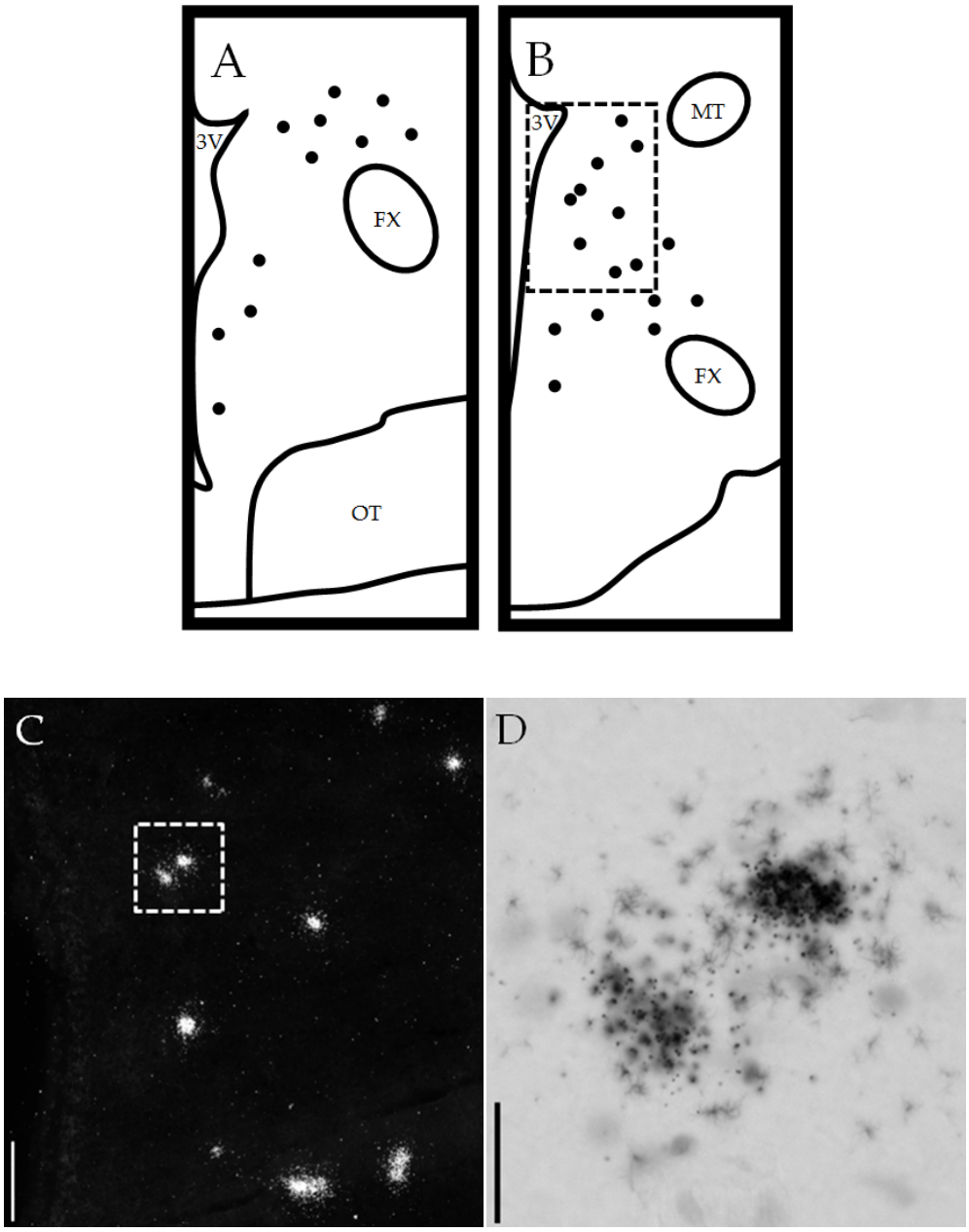

Fig. 5.1. *RFRP*-expressing cells mapped in the paraventricular nucleus (A) and dorsomedial nucleus of the hypothalamus (DMH; B) visualized by *in situ* hybridization. *RFRP*-expressing cells observed in the mare brain at low (C; 100 µm scale bar) and high (D; 20 µm scale bar) magnification within the DMH. Fornix (FX), mammillothalamic tract (MT), optic tract (OT), and third ventricle (3V).

5.4.2 Experiment 2. Adenohypophyseal expression of eGPR147 (RFRP3 receptor) during the breeding and non-breeding seasons

Adenohypophyseal expression of *GPR147* was evident during the winter anovulatory season, as well as during the follicular and luteal phases of the estrous cycle. However, expression during the winter anovulatory season did not differ (0.74 ± 0.09; $P = 0.60$) from that of either the follicular (1.11 ± 0.34) or luteal (0.88 ± 0.26) phases of the estrous cycle when normalized to a pooled reference sample.

5.4.3 Experiment 3. Effects of eRFRP3 on GnRH-mediated secretion of LH from adenohypophyseal cell culture

Adenohypophyseal cells cultured with GnRH increased ($P < 0.0001$) media concentrations of LH 7-fold (17.5 ± 1.7 ng/mL) compared to vehicle (2.6 ± 0.3 ng/mL; Fig. 5.2). However, eRFRP3 failed to reduce adenohypophyseal responsiveness to GnRH at any dose tested, as concentrations of LH in eRFRP3 + GnRH-containing media did not differ ($P \geq 0.18$; Fig. 5.2) from the positive control media stimulated with GnRH alone. Furthermore, oRFRP3 preparations previously used to suppress adenohypophyseal responsiveness to GnRH in a dose-dependent manner in ovine pituitary cultures was ineffective in equine adenohypophyseal cultures (Fig. 5.2).

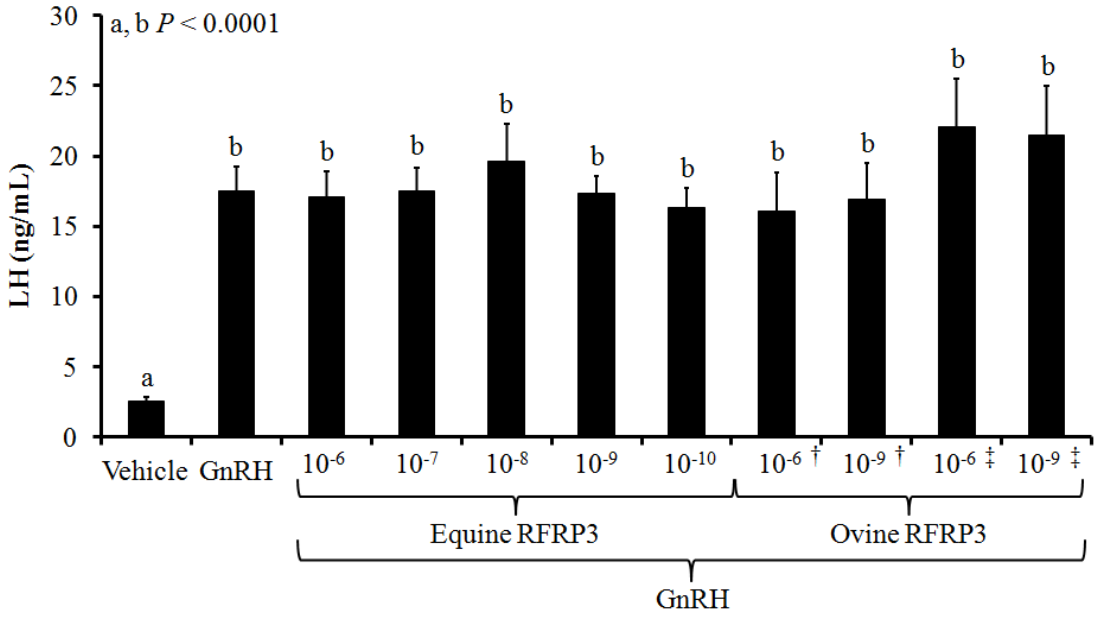

Fig. 5.2. Luteinizing hormone concentrations in culture media collected from winter anovulatory mares. Mares were fitted subcutaneously with Alzet osmotic pumps (100 µg GnRH/h for 12-18 d) to stimulate synthesis of LH by gonadotropes. Ovine RFRP3 was synthesized by: Auspep Pty, Ltd.† and United Biochemical Research, Inc.‡. Number of replicates: vehicle and GnRH n = 16; eRFRP3 n = 14-15; oRFRP3: Auspep Pty, Ltd. n = 13-15, United Biochemical Research, Inc. n = 7.

5.4.4 Experiment 4. Stimulatory effects of RF9 (RFRP3 receptor-signaling antagonist) on secretion of LH during the winter anovulatory period

Mean secretory patterns of LH observed in plasma samples collected from control and RF9-treated mares are presented in Fig. 5.3 and mean plasma concentrations of LH for pre- and post-treatment periods are presented in Fig. 5.4. On day 0, RF9 induced a robust and sustained increase ($P < 0.0001$) in circulating concentrations of LH relative to Controls (Fig. 5.3A and Fig. 5.4A). Continuous GnRH infusion at 100 µg/hr increased ($P = 0.001$) mean LH concentrations from day 0 (0.21 ± 0.03 ng/mL) to day 4 (0.33 ± 0.03 ng/mL) in Control and RF9 treated mares. On day 4, RF9 caused a

sustained increase ($P = 0.0002$) in circulating concentrations of LH relative to Controls (Fig. 5.3B and Fig. 5.4B). Intravenous injection of GnRH induced LH release in both groups, but responsiveness to GnRH as determined by area under the curve did not differ ($P = 0.91$) between Control (54.9 ± 9.8) and RF9 (57.1 ± 17.5) treatments.

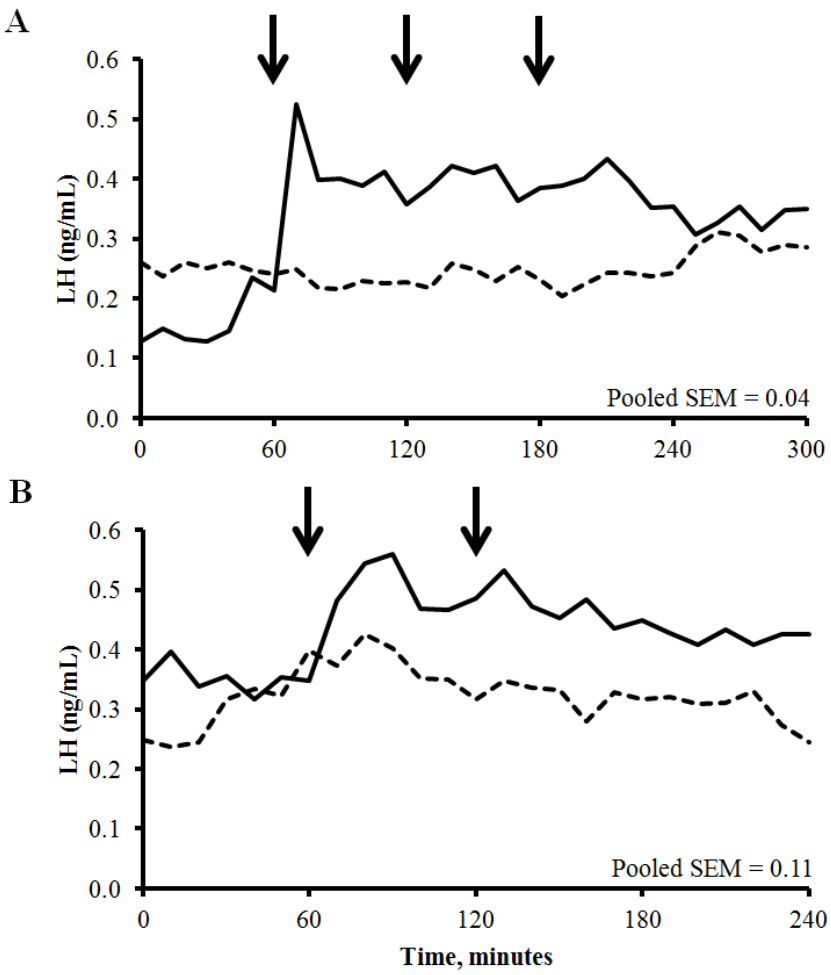

Fig. 5.3. Least squares means concentrations of LH in control (dashed line) and RF9 (solid line)-treated mares on day 0 (pre-GnRH infusion; A) and day 4 (post-GnRH infusion; B). Black arrows indicate times of saline or RF9 (0.2 or 0.4 mg/kg/injection on day 0 and 4, respectively) injections on each day of the experiment.

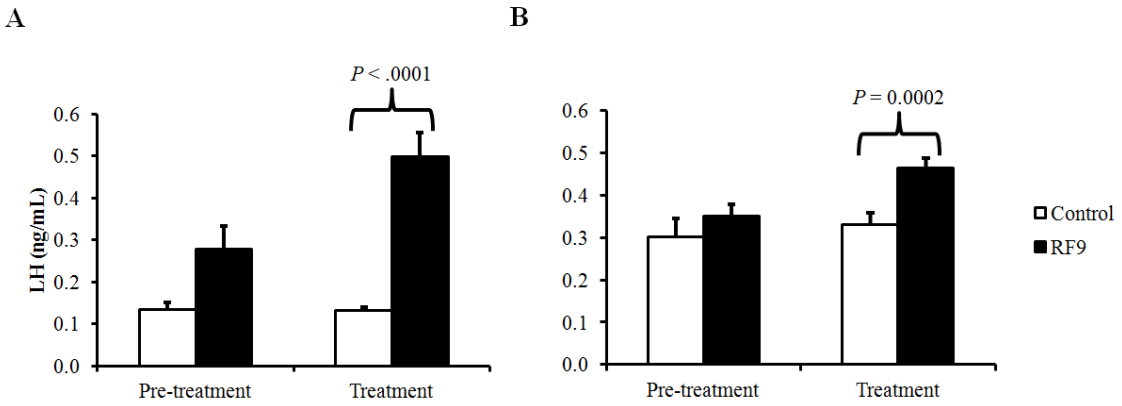

Fig. 5.4. Mean plasma concentrations of LH for pre- and post-treatment periods on day 0 (pre-GnRH infusion; A) and day 4 (post-GnRH infusion; B). The pre-treatment period consisted of blood samples collected before RF9/saline treatment, whereas the post-treatment period consisted of a 4 h sampling period on day 0 and a 3 h sampling period on day 4. Period x treatment interaction $P < 0.0001$.

5.5 Discussion

Results of experiments presented herein indicate that the genomic machinery associated with hypothalamic *eRFRP3* and adenohypophyseal receptor ligand signaling is present in the mare. However, mRNA expression for the ligand and receptor did not appear to fluctuate with season or stage of the estrous cycle. Nonetheless, a RFRP3 receptor-signaling antagonist (RF9) was shown to create robust stimulatory effects on secretion of LH during the non-breeding season which contrasted surprising inabilities of both the eRFRP3 (predicted) sequence and ovine RFRP3/GnIH to inhibit secretion of LH (Prezotto, 2012).

In initial experiments, we hypothesized that the number of neurons expressing *RFRP3* in the hypothalamus of the mare would be elevated during the winter anovulatory period and luteal phase of the estrous cycle when photostimulation and circulating estradiol concentrations are minimized. However, since changes in *RFRP3*

expression did not differ with season or reproductive phase, functional regulation of the RFRP3 system in the mare may be gated further down-stream. This could potentially include differential rates of translation, vesicular exocytosis at neuronal terminals, or receptor availability on target tissues. Both GnIH and its mammalian homologue, RFRP3 have been linked to the regulation of reproductive seasonality in other seasonal breeding species (avian, rodents, and ovine), with marked changes in GnIH/RFRP3 expression and peptide accumulation observed in the hypothalamus in response to changing photoperiod (Tsutsui et al., 2000; Ubuka et al., 2005; Clarke et al., 2008; Revel et al., 2008). During the photostimulatory periods of these species, estradiol concentrations fluctuate with follicular growth to communicate feedback to the medial basal hypothalamus. Another member of the RF-amide family, kisspeptin, acts in a contrasting manner to that of GnIH/RFRP3, and exhibits increased hypothalamic mRNA expression and protein concentration under the influence of estradiol (Kinoshita et al., 2005); however, the role of estradiol in GnIH/RFRP3 has not been fully elucidated (Kriegsfeld et al., 2006; Popa et al., 2008; Molnar et al., 2011).

As noted earlier, RFRP3 has been shown in several experiments to suppress the secretion of LH (Kriegsfeld, 2006; Clarke et al., 2008; Kadokawa et al., 2009; Rizwan et al., 2009) in rats, hamsters, sheep, and cattle, as well as to directly reduce adenohypophyseal responsiveness to GnRH, in a dose-dependent manner (Hinuma et al., 2000; Kriegsfeld et al., 2006; Clarke et al., 2008). Based on results from cell culture studies (Ciccone et al., 2004; Ubuka et al., 2006; Clarke et al., 2008; Murakami et al., 2008), RFRP3 appears to be able to regulate secretion of LH through direct inhibitory

effects on gonadotropes. In addition, *LHβ* and *FSHβ* gene expression is reduced in GnRH-stimulated ovine pituitary cells treated with RFRP3 (Clarke et al., 2008). This functionality is bolstered by anatomic data from both birds (Bentley et al., 2003; Ukena et al., 2003; Osugi et al., 2004) and mammals (Clarke et al., 2008). In sheep, cell bodies containing RFRP3/GnIH are located within the DMH and PVN, with PVN cell bodies also projecting towards the neurosecretory zone of the median eminence (Clarke et al., 2008). We have observed a similar anatomic pattern in the mare (Amstalden, Bentley and Williams, unpublished observations). However, in our experiments, adenohypophyseal expression of *GPR147/NPFF1* during the winter anovulatory season did not differ from that during either the follicular or luteal phases of the estrous cycle. Thus, the hypothesis that expression *GPR147/NPFF1* is up-regulated during the winter anovulatory season, which would serve to drive a seasonal reduction in gonadotrope responsiveness to GnRH and reduce circulating concentrations of LH, was not supported. Moreover, functional studies using primary anterior pituitary cell cultures failed to support the hypothesis that RFRP3 reduces the responsiveness of gonadotropes to GnRH in the mare, as neither equine nor ovine RFRP3 reduced secretion of LH. These results are in clear contrast to those obtained with primary adenohypophyseal cell cultures from several other species, including rats (Murakami et al., 2008), poultry (Ciccone et al., 2004), and sheep (Clarke et al., 2008).

In a subsequent series of *in vivo* experiments, Prezotto (2012) set out to determine if RFRP3, when administered peripherally, decreases circulating concentrations of LH during the follicular phase of the estrous cycle and in seasonally

anovulatory mares pre-treated with GnRH to stimulate synthesis of LH in gonadotropes. Results indicated that neither acute nor chronic administration of eRFRP3 at any dose tested, nor oRFRP3 given as a large single dose, are able to suppress episodic release, mean concentration, or GnRH-induced release of LH in mares (Prezotto, 2012). Earlier studies in male calves (Kadokawa et al., 2009) and ovariectomized ewes (Clarke et al., 2008) indicate that RFRP3 suppressed pulsatile release of LH. However, this is in contrast to other reports that demonstrate neither icv nor iv RFRP3 treatments suppress secretion of LH in ewes (Caraty et al 2012; T.M. Nett, personal communication). A similar failure of RFRP3 to suppress LH has been reported in ovariectomized, estradiol-replaced rats (Anderson et al., 2009) and ovariectomized pre-pubertal gilts (Heidorn et al., 2010). Further, stimulatory effects have been reported in the Syrian (Ancel et al., 2012) and Siberian (Ubuka et al., 2012) hamster. Therefore, the role of RFRP3 in mammals has remained controversial as a result of the variable ability of RFRP3 to suppress secretion of LH.

Importantly, an additional approach for examining the RFRP3 signaling network became available with the development of a selective antagonist for the NPFF receptors, RF9 (Simonin et al., 2006). The latter has been shown to induce a potent release of LH in rats, mice, and sheep when administered centrally or peripherally (Pineda et al., 2010; Caraty et al., 2012; Rizwan et al., 2012). In the current experiment, we tested the hypothesis that peripheral administration of RF9 induces secretion of LH in the winter anovulatory mare. Similar to results obtained in rodents and ewes (Pineda et al., 2010; Caraty et al., 2012; Rizwan et al., 2012), RF9 induced a marked increase in circulating

concentrations of LH. Furthermore, when mares were pre-treated for 72 h with GnRH to induce additional synthesis of LH in their seasonally-depleted pituitaries, the selective antagonist remained stimulatory. However, when mares were treated with a large, bolus dose of GnRH to induce maximum release of LH, responses did not differ between Control mares and those treated earlier with RF9.

Although the specific mechanisms through which RF9 stimulates secretion of LH remain to be determined, the possibility exists that it is acting directly at the level of the GnRH neuron. To date, data from all species reported (avian, rodents, ovine, sub-primates, humans, and equine) have demonstrated GnIH/RFRP3-immunoreactive fibers in close proximity to GnRH neurons (Bentley et al., 2003; Kriegsfeld et al., 2006; Ubuka et al., 2008; Smith et al., 2008; Qi et al., 2009; Ubuka et al., 2009a; Ubuka et al., 2009b; Amstalden, Williams, and Bentley, unpublished observation). Furthermore, *GPR147/NPFF1* is expressed in GnRH neurons in birds (Ubuka et al., 2008) and rodents (Rizwan et al., 2012). Functional support for this hypothesis includes the reduction in firing activity (Ducret et al., 2009; Ubuka et al., 2009b; Wu et al., 2009) and immediate early gene expression in GnRH neurons (Anderson et al., 2009) with administration of RFRP3. In addition, RFRP3/GnIH directly inhibited GnRH neuronal activity by hyperpolarizing the neuronal membrane through K+ conductance in dwarf gourami (Saito et al., 2010) allowing for the down-stream reduction in LH synthesis and secretion.

Although RFRP3 appears to function through GPR147 expressed within both the hypothalamus and pituitary (Hinuma et al., 2000; Ubuka et al., 2009b), functional

variability associated with RFRP3 effects has resulted in uncertainty regarding its mechanism of action in mammals. It is well documented that mammalian gonadotropes couple GnRH receptor with $G_{\alpha q/11}$ to stimulate phospholipase C (PLC), resulting in the generation of inositol triphosphate (IP3) and diacylglycerol (DAG; Naor, 2009). These two components then activate protein kinase C (PKC) and trigger calcium mobilization to induce phosphorylation of mitogen-activated protein kinases (MAPKs; Naor, 2009). Mechanisms proposed in mammals to transmit GnIH/RFRP3 signal at the level of the gonadotrope ultimately result in inhibiting intracellular calcium mobilization (Clarke et al., 2008), the primary GnRH receptor-induced signal for gonadotrope exocytosis (Stojilkovic and Catt, 1992; Stojilkovic and Catt, 1995), as well as phosphorylation of mitogen-activated protein kinases (ERK-1/2; Sari et al., 2009) to inhibit gonadotropin gene expression (Haisenleder et al., 1998). Therefore, GnIH/RFRP3 effectively reduces GnRH-induced gonadotropin synthesis and secretion.

In the mare, data failed to demonstrate any functional effects of RFRP3 *in vivo* (Prezotto, 2012) or *in vitro*. However, the emergence of RF9 as an antagonist of RFRP3 allows for additional methods to evaluate the RFRP3 site of action and mechanism by which RF9 functions to antagonize the RFRP3 system. The possibility exists that RF9 acts directly on GnRH neurons through GPR147 to antagonize the RFRP3 system. In fact, results indicate that the effects of RF9 are not on pituitary gonadotropes (Pineda et al., 2010). Further, the effects of RF9 are blocked by GnRH antagonists (Anderson et al., 2011; Caraty et al., 2012). In accordance with previous observations, RF9 induced a robust and sustained release of LH when administered to the mare peripherally. Further,

as secretion of LH in mares was induced rapidly, RF9 likely acts centrally in the rapid secretion of GnRH. Unlike the uncertainty regarding the functional role of RFRP3 in mammals, RF9 induces secretion of LH in all mammals studied (Pineda et al., 2010; Caraty et al., 2012; Rizwan et al., 2012) illustrating indirectly that the mammalian RFRP3 system is functional.

In conclusion, the current data indicates that the cellular machinery associated with RFRP3 function is present in the equine hypothalamus and adenohypophysis, although no evidence emerged to support a seasonal regulation of genes for the hypothalamic peptide or its receptor in the pituitary. Thus, the functional role of RFRP3 during different reproductive states in the mare remains unclear. Nonetheless, using RF9, an RFRP3 receptor antagonist, we have provided the first evidence that the RFRP3 signaling network is functional in the horse. The basis for the failure of RFRP3 itself to act in an inhibitory fashion on secretion of LH remains undetermined.

CHAPTER VI

CONCLUSIONS

The horse possesses unique reproductive endocrine features that make them both a challenge to manage as well as amenable to unorthodox therapies. This is demonstrated in research conducted under the first over-arching aim that indicates that the robustness of anterior pituitary and ovarian responses of winter anovulatory mares to continuous GnRH treatments are similar whether initiated in early February or early March in North America. However, since strategies to accelerate Julian day foaling dates are the primary concern of breeders, it would seem plausible to begin such treatments in early February rather than in March. If this approach is chosen and mares are allowed to ovulate spontaneously, without any additional gonadotropic support, it appears that continuous GnRH therapy would need to continue to the end of March in order to assure continued cyclicity in the absence of pregnancy. However, prevailing evidence indicates that continued treatment following ovulation may not be necessary if mares are induced to ovulate with additional gonadotropic support (e.g., hCG or surge-inducing treatment with GnRHa). Such a strategy would help to ensure normal luteal function and thus maintenance of pregnancy if mares are bred. In fact, continuous GnRH treatment through March was used successfully in conjunction with hCG-induced ovulation. Such treatments provide an effective means to accelerate the Julian day of pregnancy and support primary and secondary corpora luteal development and function to maintain pregnancy following treatment withdrawal while reducing the intervals to first 35 mm

follicle, breeding, ovulation, and pregnancy. However, more direct tests are needed to determine whether continuous GnRH treatment can be terminated in a normal management scenario if ovulations are induced as described above as opposed to being allowed to occur spontaneously. Nonetheless, in spite of the biological advantage of native GnRH in this setting, as shown in this dissertation, its value continues to remain unexploited from a commercial perspective. This appears to be driven mainly by the current lack of a practical and efficient delivery platform that does not require minor surgery (e.g., injectable depot delivery), an approach currently under investigation in the laboratory of Dr. Gary Williams (Texas A&M AgriLife, Beeville).

Data collected under the second over-arching aim indicate that the cellular machinery associated with RFRP3 function is present in the equine hypothalamus and adenohypophysis. This includes the presence of *RFRP3* expressing neurons in the hypothalamus, RFRP3-containing neuronal projections towards GnRH neurons and the median eminence (Amstalden, Bentley, and Williams, unpublished observations), and presence of *RFRP3* receptor in the hypothalamus (Amstalden and Williams, preliminary data) and adenohypophysis. However, the functional role of this hypothalamic peptide during different reproductive states in the mare remains unclear. Functional data regarding the site of RFRP3 action also remain unclear, as administration of the selective RFRP3 receptor antagonist (RF9) produces a robust increase in LH release in the transitional mare. These data are the first to illustrate such effects in a long-day breeder, and provide indirect evidence that RFRP3 action probably involves an inhibitory influence on GnRH release at the hypothalamic level. However, our laboratory has been

unable to demonstrate those inhibitory effects using both eRFRP3 and oRFRP3 (Prezotto, 2012) and experiments failed to support the hypothesis that RFRP3 reduces the responsiveness of gonadotropes to GnRH. Notwithstanding the lack of direct evidence for ligand functionality or seasonal changes in expression of *RFRP3* and its receptor in the mare, data presented within this dissertation using an RFRP3 receptor-signaling antagonist (RF9) provide indirect evidence that the RFRP3 system is indeed functional in this species. Future directions to elucidate the functionality of the RFRP3 system in the mare may involve determining secretory patterns and physiological concentrations of RFRP3 throughout the year. This will allow for the development of alternative techniques to better study and subsequently manipulate the RFRP3 system. Also, as neuroendocrine signaling is regulated by numerous pathways, it may prove fruitful to concomitantly administer other hormones (i.e., kisspeptin, sex steroids, etc.) with RFRP3 or RFRP3 receptor antagonists to evaluate hormone interactions and the ability of one pathway to suppress or work synergistically with another.

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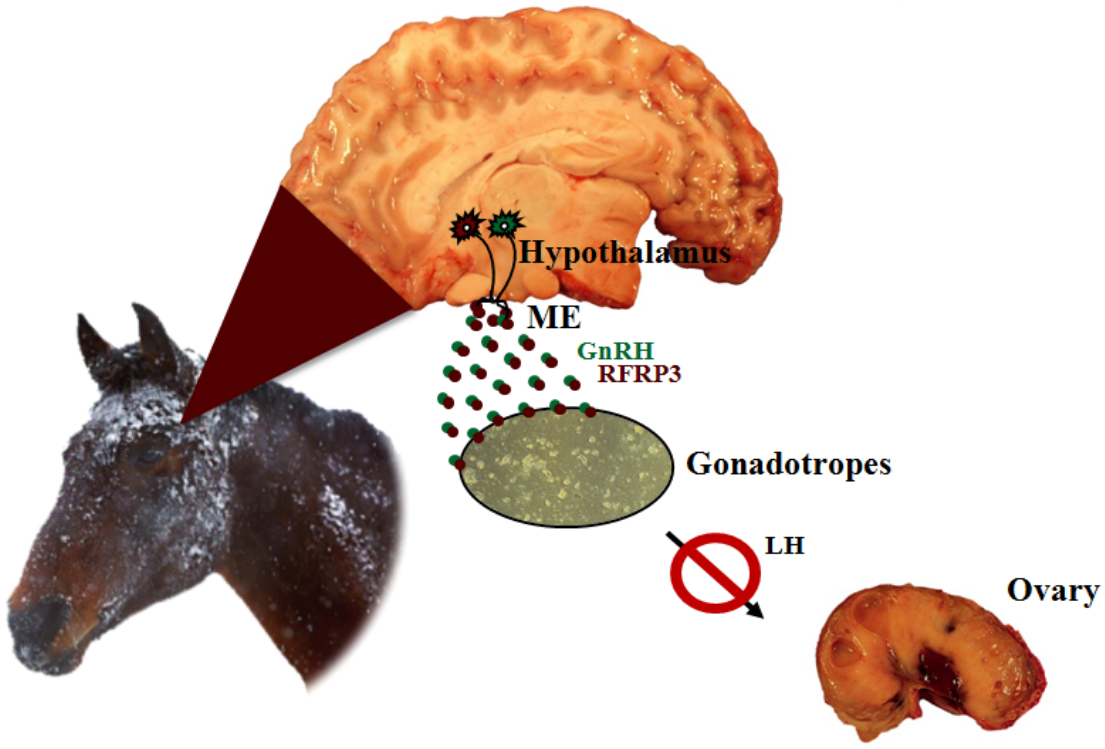
APPENDIX

THEORETICAL DEVELOPMENT AND FRAMEWORK

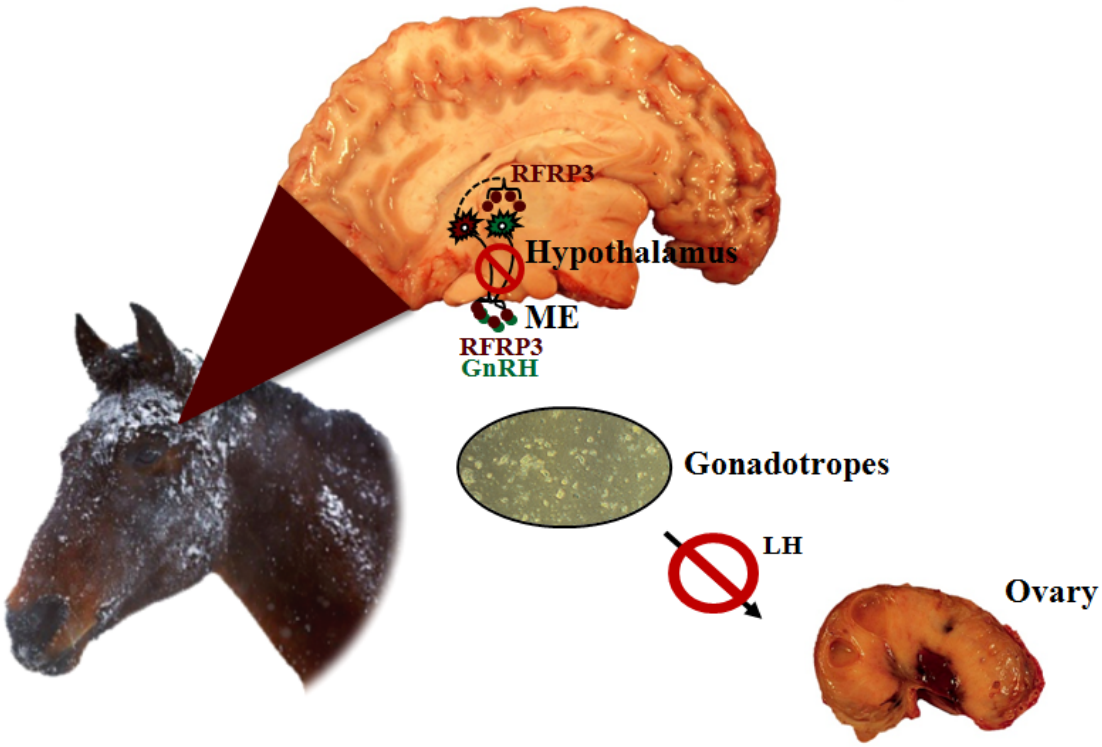
Original hypothesized model

The RFRP3-neuronal soma (maroon) are located within the dorsomedial nucleus of the hypothalamus (DMH). These neurons project terminals to the external zone of the median eminence (ME) and secrete RFRP3 (maroon spheres) into the portal vasculature that drains over the adenohypophysis. Similarly, GnRH-neuronal soma (green) are dispersed throughout the medial basal hypothalamus, releasing GnRH (green spheres) into the portal vasculature. Secreted GnRH binds to membrane-bound GnRH receptors on gonadotropes located within the adenohypophysis. During the breeding season (long days), gonadotropes secrete functional amounts of LH (black arrow) to drive follicular development and ovulation. However, during the non-breeding season (short days), RFRP3 secreted within the portal vasculature acts at the level of the adenohypophysis to reduce gonadotrope responsiveness to GnRH. This in turn reduces the synthesis and secretion of LH by gonadotropes and inhibits follicular growth and ovulation during the non-breeding season.


Hypothalamus
ME
GnRH
RFRP3
Gonadotropes
LH
Ovary

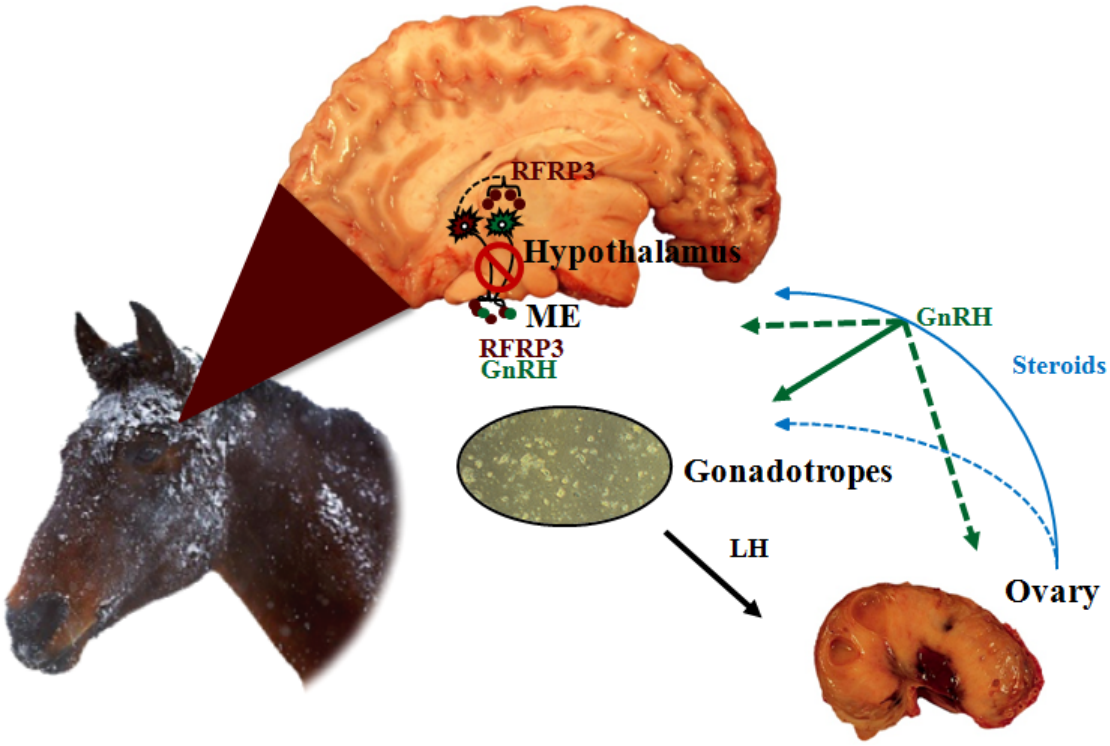
Revised hypothesized model

The RFRP3-neuronal soma (maroon) are distributed along a loose continuum from the level of the paraventricular nucleus (PVN) to the dorsomedial nucleus of the hypothalamus (DMH). These neurons project terminals to the external zone of the median eminence (ME) and towards GnRH neuronal soma and terminals (green) dispersed throughout the medial basal hypothalamus. Secretion of RFRP3 (maroon spheres) into the portal vasculature binds to GPR147, directly or indirectly inhibiting the secretion of GnRH (green spheres). Further, RFRP3 neuronal terminals have direct or indirect (dashed line) contact with GnRH soma to decrease GnRH synthesis. The culmination of this inhibition suppresses the secretion of GnRH into the portal vasculature, preventing LH synthesis and secretion (black arrow) by gonadotropes, and ultimately inhibiting ovarian activity during the non-breeding season (short days).


RFRP3
Hypothalamus
ME
RFRP3
GnRH
Gonadotropes
LH
Ovary

Merged hypothesized model

Upon exogenous infusion of a superphysiological dose of native GnRH (solid green arrow), adenohypohyseal GnRH receptors bind the hormone, LH syntheis and secretion (solid black arrow) is driven, and ovarian activity is stimulated in the winter anovulatory mare. Additionally, the superphysiological dose of native GnRH may also be acting at the level of the hypothalmus (top dashed green arrow) or ovary (bottom dashed green arrow) to drive steroidogenesis and the rapid onset of estrus behavior. One or more of these processes release the mare from the inhibition placed on the reproductive axis during the non-permisive photoperiod as depicted in the "Revised hypothesized model" above. As a result of ovarian stimulation, steroidogenesis occurs and acts systemically at the hypothalamus (solid blue arrow) to induce estrus behavior and the adenohypophysis (dashed blue arrow) to further up-regulate the synthesis and secretion of LH.


RFRP3
Hypothalamus
ME
RFRP3
GnRH
GnRH
Steroids
Gonadotropes
LH
Ovary

Additional methodology

Equine LH radioimmunoassay

1. Iodinated Product: Iodination grade eLH (AFP-5130A)

2. Antibody: Anti-equine LH (AFP-240580). Dilution 1:100,000

3. Standards (stds): Iodination grade equine LH (AFP-5130A; 0.1 – 20.0 ng/mL)

4. References (ref): equine LH added to equine serum

5. RIA Procedure:

Day 1: Begin Assay

1. NSB – 500 µl of 1% PBS-EW (egg white)
2. 0 Std – 500 µl of 1% PBS-EW
3. Stds – 200 µl std + 300 µl of 1% PBS-EW
4. Ref – 200 µl ref + 300 µl of 1% PBS-EW
5. Unknown – 200 µl unknown sample + 300 µl of 1% PBS-EW
6. Pipette 200 µl of PBS-EDTA + 1:400 NRS without primary antibody into NSB tubes only
7. Pipette 200 µl of anti-eLH (diluted in PBS-EDTA + 1:400 NRS) into all tubes except NSB and TC tubes
8. Pipette 100 µl 125I-eLH (20,000 cpm/100 µl diluted in 1% PBS-EW) into all tubes
9. Vortex tubes briefly and incubate for 24 h at 4°C

Day 2: Add Second Antibody

1. Pipette 200 µl of Sheep-anti-rabbit gamma globulin (SARGG; 2nd Ab) diluted in PBS-EDTA without NRS into all tubes except TC tubes
2. Vortex tubes briefly and incubate for 48-72 h at 4°C

Day 4: Pour Off Assay

1. Add 3 ml ice cold PBS (0.01 M; pH 7.0) to all test tubes except TC tubes
2. Centrifuge tubes for 1 h at 4°C at 3600 rpm
3. Decant supernatant
4. Count radioactivity of each tube using a gamma counter

Progesterone radioimmunoassay

Single Antibody RIA Kit, Diagnostic Products Corporation, Los Angeles, CA

1. Iodinated Product: Iodination grade hP4

2. Antibody: Anti-human P4 coated tubes

3. Standards: Human serum with added P4 (0.1 – 20.0 ng/mL)

4. Reference: Human standard preparation added to bovine serum

5. RIA Procedure:

    Day 1: begin and complete assay

        1. Pipette in non-coated polypropylene tubes

            NSB – 100 µl of 0 Std

        2. Pipette in antibody coated tubes

            0 Std – 100 µl

            Std – 100 µl

            Ref – 100 µl

            Unknowns – 100 µl

3. Pipette 1 ml of ^{125}I –P4 provided in the kit to all tubes including two Total Count non-coated polypropylene tubes

4. Vortex tubes briefly and incubate at room temperature for 3 h

5. Pour off supernatant

6. Count radioactivity of each tube using a gamma counter

Primary equine adenohypophyseal cell culture

Collection

1. Remove the frontal bone cap to expose the brain using a bone saw
2. Tilt the head caudally and slowly remove the brain, cutting meninges, cranial nerves, and the infundibulum (close to the pituitary) to expose the pituitary gland
3. Using a scalpel blade, make four cuts through the meninges around the pituitary gland
4. Using a forceps, grasp a corner of the meninges and withdrawal the pituitary gland from the sella tursica
5. Place the pituitary in a whirl-top bag and place on (not in) ice for transport to the lab

Tissue Processing

1. Place pituitary gland in a sterile pitri dish and rinse with 1X DMEM
2. Utilizing a sterile scalpel blade, forceps, and scissors, remove the meninges and neurohyphoshesis
3. Place pituitary gland in sterile pitri dish with 1X DMEM and mince well (tissue should be mushy)
4. Rinse tissue 3X with 1X DMEM (~20ml/rinse/pituitary) using a scalpel blade as a guard to prevent tissue loss into a sterile beaker
5. Place tissue in a sterile Erlenmeyer flask containing 75ml of 0.3% Collagenase Solution and incubate in a 37°C water bathe while gently stirring with a magnetic stir bar for 90 minutes

6. Filter dispersed cells through double thickness of sterile gauze into a sterile 150ml beaker
7. Transfer cell suspension to 50ml sterile conical tubes and centrifuge for 15 minutes at 1000 x g
8. Decant supernatant and resuspend pellets with 1X DMEM using a 10 ml pipette
9. Pool the suspension and bring to 50 ml, centrifuge at 1000 x g for 15 minutes
10. Remove supernatant, resuspend pellet (3X)
11. Resuspend pellet with 20 ml 10% Serum Media
12. Perform cell counts using a hemacytometer and dilute to 300,000 cells/mL with 10% Serum Media

Cell Plating and Treating

1. Incubate plated cells in 10% Serum Media in 6-well plates for 96 hours
2. Replace media with fresh 10% Serum Media at hours 48 and 72
3. Replace media with 1x DMEM media + L-glutamine at hour 96
4. Wash plated cells with 1x DMEM media + L-glutamine at hour 120 twice
5. Apply treatment in 1x DMEM media + L-glutamine
6. Collect media 4 hours post treatment initiation and store in microcentrifuge tubes at -20°C

Reagents

1X DMEM (500 ml)	Volume
100X MEM non-essential amino acids 10 mM	5 ml
50X MEM amino acids w/o L-glutamine	5 ml
Fungizone (amphotericin-B 250 µg/ml)	0.5 ml
Pen-Strep (Pen 10,000 U/ml, Strep. 10,000 µg/ml)	0.5 ml
$NaHCO_3$ 44 mM	1.85 g
HEPES 15 mM	1.85 g
1X Dulbecco's Modified Eagle Medium	bring to 500 ml

pH solution to 6.8, filter w/0.2 µm sterile filter into sterile bottle

0.3% Collagenase Solution (75 ml)	Volume
Collagrenase Type L	0.225 g
1X DMEM	bring to 75 ml

Filter w/0.2 µm sterile filter into sterile bottle

10% Serum Media (100 ml)	Volume
1X DMEM	89 ml
L-glutamine	1 ml
Fetal Bovine Serum	10 ml

Filter w/0.2 µm sterile filter into sterile bottle

Isotopic in situ hybridization of frozen sections

Day 1 – Hybridization

Dry Slides	10 min	55°C
4% PFA in 0.1M PB	15 min	RT
0.1M PB	2 X 5 min	
Protienase K (0.5 µg) in 0.5 mM EDTA, 50 mM Tris-HCL	30 min	37°C
0.1M PB	1 X 5 min	RT
4% PFA in 0.1M PB	5 min	RT
0.1M PB	1 X 5 min	
Dip in RNase Fee Water	2 X	
Dip in 0.1 M TEA	1 X	
0.1 M TEA + Acetic Anhydride	1 X 10 min	250ul/100ml TEA; add to dish first, then TEA
2X SSC	1 X 5 min	
70%	1 X 3 min	
95%	1 X 3 min	
100%	1 X 3 min	
Chloroform	1 X 5 min	
100%	1 X 3 min	
95%	1 X 3 min	
70%	1 X 3 min	
Air Dry Slides		
Pre-Hybridization	1 hrs	37°C

1. Denature radiolabeled probe (1 X 10^6 cpm/150µl) diluted in hybridization buffer containing 100 mM DTT (with DTT added fresh from 1M stock) at 70°C for 10 min
2. Let hybridization solution cool on ice for 5 min
3. Add 200µl of hybridization solution to each slide
4. Cover with parafilm coverslip on each slide - AVOID BUBBLES

5. Hybridize overnight at (55°C) in humidified chamber containing Whatman 3MM paper wetted with 50% formamide + 5X SSC

Day 2 – Washing

1. Remove parafilm coverslips from slides and place in 5X SSC without letting slides dry

5X SSC with 10mM βME	30 min	55°C
1X TEN	3 X 10 min	37°C
RNase (10µg/ml) in 1X TEN	30 min	37°C
1X TEN	30 min	37°C
2X SSC with 10mM βME	30 min	55°C
2X SSC with 10mM βME	15 min	55°C
0.1X SSC with 10mM βME	15 min	55°C
0.1X SSC	15 min	RT
70% ETOH + 0.3M NH_4Ac	2 X 5 min	RT
95% ETOH + 0.3M NH_4Ac	3 min	RT
100% ETOH	2 X 2 min	RT

2. Air dry Slides for (1-3 hr at 37°C)

3. If needed, Expose slides to film overnight to estimate autoradiography time

Autoradiography

1. Warm photographic NBT2 emulsion in a light tight container at 44°C in a water bath (approx 45 min)

2. Warm equal volume of ddH_2O to 44°C in a water bath (approx 45 min)

3. Place a dipping chamber into water bath

In the Dark (with safe-light)

1. Mix photographic NBT2 emulsion with water in a 1:1 dilution

2. Pour emulsion into the dipping chamber and let equilibrate to 42-44°C (avoid bubbles)

3. Dip each slide once and withdrawal it slowly (keep the same motion)

4. Keep slides vertical and wipe excess emulsion from the back of the slide

5. Let slides to stand up in a rack and air dry for 2-3 hr (place slide rack in a light-tight box)

6. Place slides in a plastic slide box with desiccant

7. Wrap slide box in double foil to avoid light exposure and place at 4°C for appropriate exposure time

Developing Slides

1. Allow slides to warm to room temperature

2. Chill Kodak D-19 Developer to 15°C (in fridge or on ice) and dilute 1:1

In the Dark (with safe-light, at least 1.5 m from slides)

1. Place slides into chilled D-19 Developer (diluted 1:1) for 4 min – dip approx 10 times/min (DO NOT use developer for more than on rack)
2. Dip in H_2O for 30 sec to stop development
3. Place slides into Kodak Fixer (with hardener, diluted 1:7 with H_2O) for 5 min
4. Dip in H_2O for 5 min (can turn lights on)

Coverslipping (counterstain if needed then cover slip)

1. Dehydrate sections through ETHO
   - 70% ETOH for 1 min
   - 95% ETOH for 1 min
   - 100% ETOH for 5 min
2. Place slides in Citrosol 3 times for 5 min each
3. Cover slip with DPX

Reagents

DEPC-treated H_2O (diethilpyrocarbonate H_2O)

ddH_2O	1 L
DEPC	1 ml

Shake well until foam forms
Incubate overnight at 37^o C
Autoclave

1 M Phosphate Buffer (10X PB; pH 7.3) – RNase free

Sodium phosphate dibasic	54.65 g
Sodium phosphate monobasic	15.9 g

Depc H_2O to final volume of 500 ml

0.1 M PB	50 ml	300 ml
1 M PB	5 ml	30 ml
ddH_2O	45 ml	260 ml

4% Paraformaldehyde (pH 7.2-7.4)	100 ml	300 ml
Paraformaldehyde	4 g	12 g
Sodium Hydroxide	~3g	~10g
0.1M PB	100 ml	300 ml

*Add Sodium Hydroxide to a portion of PB to dissolve first, then add Paraformaldehyde
*This is just a staring amount of Sodium Hydroxide, add slowly until Paraformaldehyde has completely dissolved
Adjust pH then add remaining PB

1 M Triethanolamine (10X TEA; pH 8) – RNase free

Triethanolamine	1L	500 ml
(98%; 149.2 MW; density 1.124 g/ml)	135.5 ml	67.9 ml
Sodium Chloride	81.8 g	40.9 g

TEA with acetic anhydride	50 ml	300 ml
0.1 M TEA	50 ml	300 ml
Acetic anhydride	125 ul	750 ul

1M Tris-HCl (pH 8) – RNase free

Tris-HCl	78.8 g

Adjust pH with HCl
DEPC H_2O to final volume of 500 ml
Autoclave

0.5mM EDTA, 50mM TRIS-HCL	100 ml
0.5M EDTA	100 ul
1 M TRIS-HCL	5 mL

DEPC water to 100ml
0.5mM EDTA, 50mM TRIS-HCL with 0.5 µg of Protenaise K

Protenaise K plus EDTA, TRIS-HCL	50 ml
Protenaise K	1.25 ul
0.5mM EDTA, 50mM TRIS-HCL	to 50 ml

20X Sodium Chloride Sodium Citrate (20X SSC) – RNase free

Sodium Citrate	88 g
Sodium Chloride	175 g

DEPC H2O to final volume of 1 L
Autoclave

5X SSC – 1 L
250 ml 20X SSC
750 ml DEPC ddH2O

2X SSC – 1 L
100 ml 20X SSC
900 ml DEPC ddH2O

0.1X SSC – 1 L
5 ml 20X SSC
995 ml DEPC ddH2O

10X Tris-EDTA-Sodium Chloride (TEN; 1L) – RNase free

NaCl	292.2 g
Tris-HCl	100 ml 1 M Tris-HCl (pH 8)
EDTA	100 ml 0.5 M EDTA (pH 8)
DEPC H_2O to final volume of 1 L	

RNase A (10 ug/ml) in 1X TEN	50 ml
RNase A (20 ug/ml)	25 ul
1X TEN	to 50 ml

Pre-hybe 4X SSC, 50% formamide	50 ml	300 ml
20X SSC	10 ml	60 ml
Deioniezed Formamide	25 ml	150 ml
DEPC water	15 ml	90 ml

0.5 M EDTA (pH 8) RNase free

Ethylene diamine tetraacetate	93.05 g
NaOH pellets	12g

DEPC H_2O to final volume of 500 ml
Warm to dissolve – must be at pH 8 to stay in solution
Adjust pH with NaOH pellets
Autoclave

5M NaCl	500 ml
NaCl	146.1g
ddH_2O	to 500 ml

Autoclave

3 M Sodium Acetate, pH 5.2	100 ml
Sodium Acetate	40.81 g
RNase Free water	80 ml
pH with glacial acetic acid	
RNase Free water	to 100 ml
Autoclave	

1M DTT	10 ml
DTT	1.545 g
0.01M Sodium Acetate pH 5.2	10 ml
Filter sterilize, and make 1 ml aliquots	
Store at -20°C	

5M NH_4AC	500 ml
NH_4AC (FW 77.08)	192.7g
ddH_2O	to 500 ml

TE pH 8.0	1 L
1M Tris, pH 8.0	10 ml
0.5M EDTA, pH8.0	2 ml
ddH_2O	to 1 L

Hybridization Buffer (RNase free)	Final	Make 50 ml
Deionized Formamide	50%	25 ml of 100%
NaCl	0.3 M	3 ml of 5 M stock
Tris-HCl (pH 8)	20 mM	1 ml of 1 M stock
EDTA (pH 8)	5 mM	0.5 ml of 0.5 M stock
$NaPO_4$ (pH 8)	10 mM	0.5 ml of 1 M stock
Denhardt's solution	1X	1 ml of 50X stock
*Dextran Sulfate	10%	5 g (dilute in H_2O before adding)
Yeast tRNA	0.5 mg/ml	2.5 ml of 10 mg/ml stock
DEPC H_2O		q.s. (to final volume; ~ 10 ml)

*Add ~10ml of water to conical tube, then add Dextran Sulfate. Mix this first before adding other reagents.
Before use, add 1 M DTT to a final concentration of 100 mM DTT for ^{35}S-labeled probes

Yeast tRNA (10mg/ml)
Yeast tRNA (25mg)
Dilute 25mg (one vial) in 2.5 ml sterile water
Incubate for 1 hour at RT
Aliquot and Store at -20˚C

50X Denhardt's Solution (RNase free)	Final	Make 100 ml
Ficoll 400	1%	1 g
Polyvinylpyrrolidone	1%	1 g
BSA (Fraction V)	1%	1 g
DEPC Water		q.s. (to final volume)

RNase A
RNase A, 50mg vial one vial
TE 2.5 ml
Mix in vial then aliquot
Store at -20˚C

Cresyl Violet
5 g cresyl violet (Sigma C-1791)
0.5 g sodium acetate
Dissolve in approximate 800 ml ddH_2O
Adjust pH to 3.5 with glacial acetic acid
Adjust to final volume of 1 L
Filter
Adjust pH to 3.13 to 3.14 with acetic acid
* Obs: cresyl violet solution should be filtered before each use

10X Acetate Buffer
2.5 g sodium acetate (MW 136.08)
Dissolve in approximately 450 ml dd H2O
Adjust pH to 3.5 with glacial acetic acid (approx 20 ml)
Adjust final volume to 500 ml

Acidified 70% Ethanol
10 ml acetic acid
490 ml 70% Ethanol

All solutions and dishware must be RNase Free