

**ROLE OF AN EQUINE HOMOLOGUE OF GONADOTROPIN-INHIBITING  
HORMONE IN CONTROLLING SECRETION OF LUTEINIZING HORMONE  
IN THE MARE**

A Thesis

by

LIGIA DIAS PREZOTTO

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

MASTER OF SCIENCE

May 2012

Major Subject: Physiology of Reproduction

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Approved by:

Co-Chairs of Committee,	Gary L. Williams
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**ABSTRACT**

Role of an Equine Homologue of Gonadotropin-Inhibiting Hormone in Controlling  
Secretion of Luteinizing Hormone in the Mare. (May 2012)

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Four experiments were conducted to test the hypothesis that RF-amide related peptide 3 (RFRP3) negatively regulate the secretion of LH in mares. In Exp. 1, mares received native gonadotropin-releasing hormone (GnRH) continuously at a rate of 20  $\mu\text{g/h}$ , delivered subcutaneously using Alzet osmotic pumps during the luteal phase of the estrous cycle. Mares were treated with i.v. bolus injections of 0, 500 and 1,000  $\mu\text{g}$  eRFRP3 on days 4, 6 and 8 of cycle. Mean concentrations of LH in the peripheral circulation averaged  $1.2 \pm 0.2$  ng/mL and did not differ among groups before or following RFRP3 treatment. In Exp. 2, pituitary venous effluent was sampled for characterization of episodic release of LH. Mares received either saline or eRFRP3 (250  $\mu\text{g}$ ) i.v. every 10 min for 6 h beginning 2 h after onset of sampling. At hour 6, each mare was challenged with 1 mg GnRH. Neither mean ICS concentrations of LH ( $1.3 \pm 0.2$  ng/ml), nor frequency ( $3.6 \pm 0.55$  episodes/h), amplitude ( $0.2 \pm 0.03$  ng/ml), or duration ( $36.3 \pm 3.5$

min) of individual secretory episodes, differed between groups before or after eRFRP3 treatment. Area under the GnRH-induced LH curve (arbitrary units) also did not differ between control and RFRP3 treated mares ( $175.9 \pm 11.4$  vs.  $192.6 \pm 10.6$ ). In Exp. 3, winter anovulatory mares (n=6) were treated continuously for 7 d with GnRH (100  $\mu$ g/h) to stimulate synthesis of LH and increase circulating concentrations of LH to values similar to the breeding season. The ICS was catheterized for blood sampling and mares were treated with saline or RFRP3 (5 mg) in a replicated Latin square design. Treatment with RFRP3 failed to alter ICS mean concentration of LH ( $0.95 \pm .03$  ng/ml). Finally in Exp. 4, mares in the follicular phase of the estrous cycle were assigned randomly to receive either saline (n=3) or 10  $\mu$ g/kg BW of oRFRP3 (n=3) in a single injection. No effect on mean concentration of LH was observed. In contrast to observations in birds and other mammals, results of the current experiments fail to provide evidence for functional activity of eRFRP3 or oRFRP3 in regulating LH release in the mare.

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## CHAPTER I

### INTRODUCTION

The natural breeding season of the mare occurs during long days. Therefore, in the northern hemisphere, it is generally restricted to the interval between April and October. After this period, a majority of mares become anovulatory and ovaries remain quiescent throughout the winter. Most research in this area has focused on the roles of day-length [1], pineal melatonin [2], and gonadotropin releasing hormone (GnRH) secretion [3]. However, the neuroendocrine basis of equine reproductive seasonality has not been completely elucidated.

The use of artificial lighting to extend day-length and hasten onset of spring transition is well-documented. It is an effective managerial strategy used by many large farms; however, the method is not widely utilized by smaller breeders because of limitations in facilities [4].

Pineal melatonin release, which increases in response to decreased photoperiod, has long been implicated in the control of seasonal reproduction through potential effects on secretion of GnRH [2]. There is some evidence that melatonin reduces GnRH content of the hypothalamus in mares [5] but a direct effect of melatonin on hypothalamic secretion of GnRH has not been demonstrated. Therefore, this area of investigation requires much additional work.

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This thesis was written in the format and style of *Domestic Animal Endocrinology*.

Gonadotropin-releasing hormone is the primary regulator of both LH and FSH synthesis and secretion [6, 7, 8], and existing dogma indicates that the winter anovulatory state in mares is a consequence of reduced secretion of GnRH. Therefore, some studies have focused on pulsatile [9, 10] and continuous [9, 11, 12] administration of GnRH to induce follicular growth and ovulation in mares during the anovulatory state. Although, such treatments have clearly demonstrated their ability to stimulate ovarian activity successfully, studies conducted in our laboratory have questioned whether seasonal anovulation is controlled only by limitations of endogenous GnRH secretion, or involves changes in pituitary responsiveness. Using the intercavernous sinus (ICS) cannulation technique to measure secretion of GnRH, Cooper reported no change in GnRH release during 4 different seasons [13]. This observation would support the hypothesis that winter anovulation in the mare is mediated by changes in gonadotrope responsiveness.

In 2000 Tsutsui et al. [14], reported the identification of a novel hypothalamic neuropeptide with a C-terminal LPLRF-amide sequence in the quail brain. This neuropeptide inhibited gonadotropin release in birds both *in vivo* and in cultured anterior pituitary cells. The functional RF-amide was named, gonadotropin-inhibiting hormone (GnIH). Although, other neuropeptide (e.g. endogenous opioid peptide (EOP) are known to project to the median eminence (ME) and to the posterior pituitary, and inhibit LH release by direct actions in the pituitary [15, 16], their physiological relevance regarding the control of gonadotropin release is controversial. Thus, GnIH became the most

physiologically relevant hypothalamic peptide shown to inhibit gonadotropin release in vertebrates and has been widely studied in this regard.

Following its discovery in the avian brain, studies were conducted in other species, such as mammals, amphibians, and fish [17, 18, 19, 20, 21, 22]. These studies resulted in the finding of similar GnIH-related peptides broadly termed RF-amide related peptides (RFRP). All of these RFRP possess a LPXRF-amide sequence in the C-terminus, similar to that observed in the avian GnIH [19, 23, 24, 25, 26, 27]. The RFRP gene encodes more than one peptide (i.e. RFRP1, RFRP2, and RFRP3). However, the RFRP3 appears to have the most potent effects on secretion of LH [19, 28].

Initial work in birds focused on the possibility that the main effects of GnIH were at the hypothalamic level through effects on GnRH neurons [14, 17, 29, 30, 31]. However, studies in sheep were not able to confirm a decrease in GnRH concentrations in portal blood plasma after GnIH administration. Whereas, some investigators were able to report effects on GnRH expression in hypothalamic cells after giving RFRP3 through intracerebroventricular (ICV) injections [32], *in vivo* studies mainly measured dose-related reductions in secretion of LH after injection of GnIH/RFRP3 [18, 20, 29, 33, 34, 35].

The potential role of RFRP3, the mammalian homolog of avian GnIH, in equine reproductive seasonality has not been elucidated. Because of the economic importance of the horse and its profound seasonality, it is essential that these studies be undertaken. The objectives of the studies described herein were to determine 1) whether eRFRP3 have the ability to decrease mean concentrations of LH in peripheral plasma of mares in

the luteal phase of the estrous cycle during concomitant infusion of GnRH, 2) if continuous infusion of eRFRP3 has the ability to suppress the pulsatile release of LH in the intercavernous sinus (ICS) of mares during the follicular phase, 3) if eRFRP3 injected into anovulatory mares treated continuously with GnRH (i.e. synthesis and release of LH is similar to what is observed during breeding season) results in a suppression of LH secretion, and 4) if ovine oRFRP3 injected into mares during the follicular phase suppresses LH secretion in the ICS. The overall hypothesis is that either eRFRP3 or oRFRP3 will disrupt the normal pattern of LH secretion as detected in the peripheral circulation or in the ICS effluent.

## CHAPTER II

### REVIEW OF LITERATURE

#### **2.1 Overview of reproduction in the mare**

Mares are seasonal long-day breeders with a natural breeding season extending on average from April to October in the Northern Hemisphere. Approximately 85% of mares become anovulatory during the late fall and winter. Resumption of ovarian cycles can occur as early as February or March and as late as May or June [1]. Around the time of the autumn equinox a reduction in synthesis and release of anterior pituitary luteinizing hormone (LH) occurs, followed by cessation of ovulatory cycles in most mares [36].

Continuous infusions of GnRH into mares from mid-January or later can effectively induce secretion of LH and follicle development in winter anovulatory mares [11, 37]. These results imply that a reduction in secretion of GnRH is the likely limiting factor during seasonal anovulation. In addition, the ability of pituitary gonadotropes to respond to GnRH changes as the winter anovulatory period progresses, with a lesser response in late February compared to late December [11, 38]. However, it is not known whether this occurs as a result of innate changes in gonadotrope responsiveness to GnRH or due to chronic inactivity of cellular machinery following an extended period of limited exposure to GnRH.

The measurement of pulsatile release of both GnRH and LH in the mare can be made using the technique of ICS cannulation developed by Irvine [39], which allows



sampling from the anterior pituitary draining vein effluent. More recently, the technique has been used by Cooper et al. [13] and Velez et al. [11] in our laboratory. In the former case, it was observed that the marked decline in secretion of LH in winter was not accompanied by similar declines in ICS concentrations or pulsatile release of GnRH [13]. However, these data remain controversial and additional critical research steps are necessary in order to confirm their accuracy.

## **2.2 Potential roles for seasonality in reproduction of the mare**

### *2.2.1 Natural and artificial photoperiods*

It is well accepted that photoperiod is a major regulator of seasonality in horses and, over the years, investigations have focused on its different physiological effects. Regardless of whether lighting conditions are considered inhibitory or stimulatory, mares exposed to constant lighting conditions become refractory to that signal and their circannual reproductive rhythms resume [40, 41, 42]. This can be overcome by exposing the animal to periods of light and dark at regular intervals. Mares exposed to artificial lighting that extends day-length to 15-16 h during the winter and early spring exhibit an accelerated onset of the breeding season, which reinforces the influence and advantage of artificial lighting [43]. However, mares exposed continuously to 16 h of light and 8 h of darkness eventually return to anestrus [40, 41, 42].

Several early studies utilized different combinations of light and dark periods with the objective of determining which would induce ovulation in anovulatory mares [41, 44, 45, 46, 47]. When intact anovulatory mares were exposed to increasing photoperiod using artificial lighting, the first ovulation occurred earlier [48]. The

approaches used different combinations of light and dark periods in an effort to optimize the hastening of ovulation [40, 41, 42, 43, 44, 45, 46, 47]. However, variations in the circadian rhythms of animals in experimental conditions mimicking the perception of sunrise and sunset, which is important for the placement of the photosensitive phase, are different to those compared to natural conditions. Thus, it can be concluded that it is not possible to completely duplicate this situation under experimental conditions [49].

### *2.2.2 Importance of nutrition*

The influence of body condition score on the average interval to first ovulation of the breeding season [50] becomes apparent when mares are supplemented with high concentrate diets. Mares ovulated earlier in the spring transition under this regimen and when BW gain was promoted during the early spring [51, 52]. The combination of diet supplementation and artificially extended photoperiod also positively influence the onset of reproductive activity in the spring [53]. Moreover, mares in low body condition exposed to a high-dietary energy intake and improving body condition during spring transition had a shortened interval to first ovulation [54]. Mares grazing on pasture with grass of high quality exhibit a similar phenomenon [55].

### *2.2.3 Role of melatonin*

Melatonin is a key hormone controlling seasonal reproduction in mammals. Studies demonstrating that the pineal gland translates photoperiodic signals received by the eyes to other endocrine effects through changes in melatonin secretion were important for our current understanding of mechanisms regulating seasonal reproduction in horses and other mammals [56, 57, 58]. Production of melatonin from pineal explants

collected throughout the year is greater in December and January and returns to lower, breeding season values by February [43, 57, 59]. This observation corresponds to similar changes in concentrations of hydroxyl-indole-o methyl transferase (HIOMT), the enzyme involved in the synthesis of melatonin in the pineal [59]. The pattern and duration of melatonin secretion can be changed when mares are exposed to artificial light during the natural dark phase [44, 57].

Mares which were pinealectomized and exposed to an extended photoperiod did not exhibit hastening of reproduction transition [60], and treatment with melatonin results in a similar effect [44, 57]. In addition, mares treated with melatonin implants did not exhibit any ovarian effects when implanted on the shortest day of the year, but onset of the breeding season was advanced when treatments were applied close to the summer solstice [58]. Exogenous melatonin has been shown to affect hypothalamic concentrations of GnRH when injected in ovariectomized mares during breeding season and in sheep [61, 62]. However, conclusions drawn from such studies appear to be in conflict with others in which melatonin seemed to affect circannual rhythms rather than reproductive activity directly [49].

#### *2.2.4 Patterns of LH and GnRH secretion*

Several studies have provided evidence that the photoperiodic regulation of equine seasonal reproduction is steroid-independent [63, 64]. The secretion of LH in intact and ovariectomized mares has been shown to follow a seasonal pattern, with increased secretion during the breeding season and decreased secretion during the anovulatory period [65, 66, 67, 68]. However, there does not appear to be a similar

pattern of change for follicle stimulating hormone (FSH) [67]; although, minor changes have been observed [38]. Mares injected with GnRH during the transition period from the anovulatory phase into the breeding season had release of LH and FSH stimulated [69].

During the spring transition period, before first ovulation of the breeding season, mares often exhibit prolonged periods of estrous behavior in the absence of significant ovarian activity. However, mean circulating concentrations of LH remain relatively low until 6-7 days before the first ovulation [66, 67].

A reduction in hypothalamic content of GnRH in anovulatory mares is correlated with low stores of LH in the anterior pituitary during late winter [70]. However, the restoration in GnRH concentrations has been shown to occur earlier in the spring than restoration of pituitary LH. Maximal adenohypophyseal concentrations of LH are not observed, until mid-breeding season [70, 71]. Additionally, there appear to be differences in hypothalamic distribution of GnRH immunoreactivity during the breeding and non-breeding seasons and, in spite of findings in our own laboratory, there is evidence that the release of GnRH during the anovulatory season is reduced [71]. In the horse and some other species, LH response to exogenous GnRH is used to estimate pituitary responsiveness to endogenous GnRH. Mares in different reproductive states received different doses of GnRH which induced pulsatile LH secretion comparable to what is observed endogenously, implying that the differences observed in concentration of LH between seasons is due to a difference in endogenous GnRH secretion [72]. Moreover, a difference in overall mean secretory rate of GnRH among groups in their

breeding and non-breeding seasons was observed using the push-pull perfusion technique [73].

In sheep, estradiol plays a definitive role in reproductive seasonality. During the non-breeding season, there is an increase in the negative feedback sensitivity to estradiol through activation of the dopaminergic system and consequent suppression of the pulsatile release of GnRH and LH [74]. To the contrary, when mares were treated chronically with estradiol during nonbreeding season, mean concentrations of LH increased [64]. This indicates that winter anestrus is independent of estradiol negative feedback in the mare.

#### *2.2.5 Thyroid hormone*

Thyroid hormones have been demonstrated to contribute to the regulation of seasonal breeding in some species, such as sheep [75]. Thyroidectomy abolishes the reduction in episodic release of LH that occurs in intact ewes at the end of the breeding season. However, secretion of LH was similar in intact and thyroidectomized ewes during the breeding season [75]. The requirement for thyroid hormones occurs only during a small window of time at the end of the breeding season in ewes [76]. The secretion of LH can be inhibited in ewes thyroidectomized late in the breeding season and then treated centrally with thyroxine (T<sub>4</sub>) [77]. The role of thyroid hormones in the mare has not been extensively studied. Circulating concentrations of thyroid hormones in horses are greatest during the winter compared to other seasons [78]. Unlike sheep [77], thyroidectomy does not alter timing of the onset of the anovulatory season in mares.

### **2.3 Circannual clock mechanisms**

The study of circannual clock mechanisms is an recognized concept based on a working hypothesis involving the existence of adult stem cells in the brain, pituitary gland, and some peripheral tissues that appear to undergo synchronized division [79]. These cells proliferate, migrate and differentiate, providing the necessary substrate for specific physiological changes over time, and have cell death as a trigger for the beginning of the following cycle. This model has been characterized in species such as primates including human [80, 81], and in several hibernating [82, 83, 84, 85, 86, 87], seasonal breeding [88, 89] and seasonal migratory [90, 91, 92] species.

No changes in the circannual rhythm have been observed when seasonal animals (birds, mammals, and insects) were maintained in a constant light and dark cycle that are different from the natural exposure to a 24 h day (e.g. 23 or 25 h d) [90, 93, 94, 95]. The ability of the nervous system to monitor photoperiodic time enables the system to detect the annual cycle of day-length and synchronizes physiological changes throughout seasons which are dependent on the endogenous circadian system. When photoperiod changes it is possible to observe activation or inhibition of seasonal responses, or alteration of endogenous circannual rhythm generators that synchronize with the environment in a long term fashion [96, 97, 98].

The suprachiasmatic nucleus (SCN) serves as a pacemaker timing the circannual rhythm including activity and sleep, body temperature, pituitary activity, among other diurnal and nocturnal rhythmicity involving melatonin activity. This occurs by influencing the timing of duration of the melatonin rhythm. This also influences the

reading of melatonin signals by the pars tuberalis (PT), one of the more well-studied targets in which circadian clock genes have been characterized [99]. The location of the PT, and the fact that it is a melatonin target tissue with a high density of melatonin receptors, makes it a specialized site for regulating the circannual clock [100, 101]. It appears to function as a regulator of long-term cycles in several physiological systems. For example, some specialized PT cells (PT thyrotropes) [102] are responsible for TSH secretion, which acts locally in the pituitary gland and regulates thyroid hormone dependent mechanisms [97, 103]. In mammals, information sent from the retina to the SCN coordinating the production of melatonin by the pineal gland in a 24 h period, is the mechanism coding photoperiod [90, 104].

The endogenous circannual rhythm, driven by specific genes, drives seasonal reproductive cycles in sheep, and these cycles are maintained even under constant photoperiod [105]. This phenomenon is termed the photorefractory response. Procedures such as pinealectomy or denervation of the pineal through superior cervical ganglionectomy can be utilized to dissociate the circannual and circadian timing systems. The operations remove the melatonin signal and thus block physiological responses to photoperiod [98, 106]. Specific clock genes controlling secretion of GnRH in the hypothalamus, unlike those in the PT, have not been characterized.

One of the limiting factors for the study of the circannual clock is length of time required to observe changes within or between fractions of a circannual cycle compared to those needed for typical neuroendocrine feedback loops [107]. The minimal time for observing these changes is dependent on cyclical tissue regeneration. The theory







































































































































