ROLES OF ESTRADIOL AND A PUTATIVE NEUROPEPTIDE FF RECEPTOR ANTAGONIST, RF9 IN REGULATING GONADOTROPIN SECRETION IN THE CYCLIC AND SEASONALLY ANOVULATORY MARE

A Thesis

by

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ABSTRACT

Seasonal reproduction is highly correlated with circulating concentrations of luteinizing hormone (LH) in mares and low peripheral concentrations of LH serve as the primary basis for ovarian quiescence during the non-breeding season. Four experiments were conducted to investigate the effects and underlying relationships of 1-adamantanecarbonyl-RF-NH2 (RF9) and estradiol in the regulation of LH secretion. During the luteal phase of the estrous cycle (Experiment 1), mares were treated with two bolus intravenous injections of RF9 (0.2 and 0.4 mg/kg, respectively) within a 1-h interval to determine effects on secretion of gonadotropin-releasing hormone (GnRH), follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Dose-dependent increases ($P < 0.05$) in both FSH and LH concentrations in response to RF9 were observed. Due to an inability to validate the GnRH radioimmunoassay, changes in secretion of GnRH could not be determined. In Experiment 2, the effect of estradiol-17β (estradiol) pretreatment on responsiveness to RF9 was examined in winter anovulatory mares. Mares received a single intramuscular injection of corn oil of 5 mg estradiol in corn oil followed by a bolus injection of RF9 18 h later. Estradiol pretreatment increased ($P < 0.05$) peak concentrations of LH in response to RF9. The half-life of RF9 in the circulation of the mare following peripheral administration was determined in Experiment 3 using liquid chromatography and mass spectrometry. Intravenously-injected RF9 was rapidly removed from the mare's circulatory system; exhibiting a half-life of approximately 40 min. Finally, the role of estradiol and its interaction with GnRH

in regulating secretion of LH in anovulatory mares during increasing photoperiod was examined. In February, mares were assigned to 1 of 4 groups and served as controls (corn oil injections) or received 1 of 3 treatments: daily i.m. injections of estradiol in corn oil for 14 d, continuous subcutaneous treatment with native GnRH for 14 d at a rate of 100 μg/h, or the estradiol and GnRH treatments in combination. Estradiol alone had no effect on secretion of LH but markedly enhanced ($P < 0.0002$) responsiveness to GnRH. Due to its short half-life, RF9 may not be useful therapeutically; however, estradiol enhances responsiveness to GnRH and may be useful in treatments designed to accelerate vernal transition.

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NOMENCLATURE

BCS	Body condition score
CL	Corpus luteum
FSH	Follicle stimulating hormone
GnIH	Gonadotropin-inhibitory hormone
GnRH	Gonadotropin-releasing hormone
GPR147	G-protein coupled receptor 147
ICS	Intercavernous sinus
LH	Luteinizing hormone
LC/MS	Liquid Chromatograph and Mass Spectroscopy
RF9	Adamantanecarbonyl-Arg-Phe-NH2
RIA	Radioimmunoassay

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION

Mares are long-day breeders, with seasonal reproductive cycles occurring as a consequence of an endogenous circannual rhythm controlled primarily by photoperiod [1]. In the Northern Hemisphere, the natural breeding season of the horse falls between April and October, with approximately 85% of standard light-horse mares becoming anovulatory around the time of the autumnal equinox [1]. However, the operational breeding season is currently initiated well in advance of the natural breeding season in most commercial horse breeding operations in North America, particularly those representing the major breed organizations such as Thoroughbred, American Quarter Horse, and Standardbred etc [2]. This has occurred because January 1 has been adopted as the official birthdate of foals in the majority of these breed registries. The basis for the advanced operational breeding season is that the earlier a foal is born in the calendar year, the more time it has to grow and mature compared to a foal born later. Results of regional Thoroughbred yearling race horse sales suggest that there is a significant economic gain by assuring that foal dates are as close to January 1 as possible. Foals born in January and February were worth an estimated 30% more and March to April foals 18% more than foals born after April [3].

A common method used by most commercial breeders to initiate ovarian cyclicity in seasonally anovulatory mares is to supplement artificial lighting, maintaining a minimum of 14.5 to 16 h of light per day, beginning 60 to 90 d prior to the

intended breeding season [1,4]. This method has proven to be an effective way of initiating the onset of ovarian cyclicity in the mare and to lengthen the operational breeding season [1]. However, individual responses can be quite variable and the costs of time, labor and infrastructure needed to implement a lighting program are significant [5]. Thus, there are economic incentives for improving our understanding of fundamental neuroendocrine mechanisms controlling reproductive seasonality in the mare and for developing novel pharmacological methodologies to consistently time the first and subsequent ovulations during the operational breeding season.

Several neuroendocrine signaling pathways involved in regulating the transition from non-breeding to breeding seasons in the mare have been described. These include changes in secretion of melatonin [6], endogenous opioidergic and dopaminergic tone [7], secretion of gonadotropin-releasing hormone (GnRH), and stimulatory effects of ovarian and placental steroid hormones on gonadotropin secretion post-foaling (foal heat) and during vernal transition. Most recently, the putative role of a novel peptide first discovered in seasonally-breeding birds, gonadotropin inhibitory hormone (GnIH) has been examined in the mare for the first time by our group [8]. In addition, we were the first to evaluate a putative antagonist, 1-adamantanecabonyl-RF-NH2 (RF9), of the receptor signaling pathways for GnIH in the mare [8]. Studies suggested that RF9 may be a prospective pharmaceutical means of manipulating seasonal reproduction based on its ability to initiate a robust increase in circulating luteinizing hormone (LH) [8,9]. However, further research is needed to investigate the biological effects of RF9, its potential value as a pharmaceutical agent, and roles of ovarian steroid hormones,

particularly estradiol, in modulating RF9's hypothalamic effects. In fact, the role of estradiol in modulating traditionally-accepted aspects of hypothalamic-adenohypophyseal function in the mare has also remained controversial, especially during vernal transition. In some studies, estradiol has been shown to be stimulatory and to interact positively with photoperiod to stimulate secretion of LH during this period [10-12]. However, other studies failed to support those findings [12].

The objectives of research reported in this thesis were to determine 1) whether RF9 stimulates the secretion of GnRH, as measured in the intercavernous sinus of the mare, in concert with secretion of follicle stimulating hormone (FSH) and LH, 2) the half-life of RF9 in the circulation of the mare following peripheral administration, 3) whether estradiol modulates hypothalamic responsiveness to RF9, and 4) the role of estradiol and its interaction with GnRH in regulating secretion of LH during increasing daylight.

CHAPTER II

LITERATURE REVIEW

2.1 Overview of Annual Reproductive Cycle

Characteristic changes associated with reproductive seasonality in the mare are indicated by the hormonal patterns throughout the year, and are accentuated by marked changes in the mean concentration of LH present in the circulation. For mares during the natural breeding season in the Northern Hemisphere, concentrations of LH begin to increase from baseline values approximately one month prior to the first annual ovulation (March-May) [13]. During this period, concentrations of circulating LH continue to increase and ovarian follicular dynamics begin to change. While follicular waves are present throughout the transition period leading up to the first ovulation, the number of follicles >20mm in diameter within each wave begins to increase during vernal transition [13]. Prior to ovulation, selection of a dominant follicle occurs at deviation and both circulating concentrations of LH and size of the dominant follicle increase rapidly until ovulation, while smaller follicles become atretic [13,14]. Maximum peak concentrations of LH in the mare occur approximately 1 to 2 d after ovulation [15]. With respect to the overall cycle, mean circulating concentrations of LH continue to increase until approximately the mid-breeding season (June-July). The majority of mares enter into a winter anovulatory period following the autumnal equinox, with circulating concentrations of LH reaching their nadir by the middle of the non-breeding season (Dec.-Jan) [1,13,16-18].

The annual reproductive cycle of the mare, or circannual rhythm, is dictated primarily by changes in photoperiod [1,5,19]. The pineal gland, acting as a transducer, translates the photoperiodic signal by altering its secretion of melatonin [19-21]. Grubaugh, et al. [6] was able to demonstrate that pinealectomized mares become non-responsive to photostimulation, creating a disconnect in the long term timing of seasonal regulation. While melatonin's mechanism of action has yet to be completely elucidated, it has been shown to alter hypothalamic release of GnRH and thus the release of gonadotropins, LH and FSH and the subsequent regulation of follicular development [13,20].

2.2 Melatonin

In short day breeders, such as sheep and goats, supplementation with melatonin has been shown to have some positive effects on reproductive performance, but has limited success in advancing the onset of estrus in seasonally anovulatory ewes [20,22-24]. As a means to hasten the onset of estrous cycles in the seasonally anovulatory mare, treatment with melatonin would not appear to be justified as it has been shown to slow the increase in LH during spring transition, ultimately extending the interval to first ovulation [25]. It should be noted, however, that continuous melatonin treatment after the summer solstice has been shown to advance the ovulatory season the following year, with the early onset being explained by short day refractoriness brought on by the early increase in melatonin [25,26]. Nonetheless, melatonin treatment seems to have little practical application in the advancement of estrus during the non-breeding season.

2.3 Opioids

Endogenous opioid inhibition of the hypothalamic-pituitary axis has also been noted in equine, as it is thought to play a role in seasonal regulation and GnRH inhibition during the luteal phase. Alexander [27] reported an increase in both amplitude and frequency of GnRH, FSH, and LH pulses during the breeding season from pituitary venous samples following a bolus i.v. injection of naloxone (0.2 mg/kg BW), an opioid antagonist, prior to ovulation. When evaluating the response of anovulatory mares to naloxone, results varied, with some groups exhibiting an immediate increase in secretion of LH following treatment [28,29], while another group showed a dose dependent increase in FSH only [30]. Sharp, et al. [31] was unable to suppress the opioid inhibition of LH with high doses of naloxone (2 mg/kg), possibly a result of a bell-shaped response curve noted in later work by Irvine [32]. Davison and co-workers suggested that mares that continue to exhibit ovulatory cycles during the non-breeding season may have reduced opioidergic tone, as they exhibited an increase in LH following naloxone treatment, similar to that of mares during the breeding season [30].

2.4 Catecholamines

Similar regulation of hypothalamic-pituitary function has been reported in response to endogenous dopamine. Melrose, et al. [33] evaluated concentrations of catecholamines in the cerebrospinal fluid (CSF) of horses by age and reproductive status and found that concentrations of dopamine were significantly greater during the anovulatory season in comparison to the ovulatory season in the mare. This information

has led to numerous investigations using dopamine antagonists to evaluate dopamine's participation in seasonal regulation of reproduction in the mare. Results were variable, with some studies showing an increase in follicular activity and a decrease in time to onset of ovarian cyclicity in the spring [34-37], and others showing no effect [38-40]. Similar variability was observed when evaluating gonadotropin response to dopamine antagonists, with no acute increases in LH reported in some studies [7,41] and long-term treatment producing an increase in mean circulating LH and FSH [35,37]. Even with this variable response in cyclicity and gonadotropic response, there does seem to be an increase in prolactin in a number of studies [34,35,40]. Recombinant porcine prolactin has been shown to shorten the days to first ovulation and induce shedding in the anestrous mare, suggesting that that dopamine antagonists may have some stimulatory effect indirectly through prolactin [42].

2.5 Gonadotropin-Releasing Hormone

The central dogma of gonadotropic hormone regulation involves the anterior pituitary release of LH and FSH in response to hypothalamic secretion of GnRH. This suggests that the seasonal regulation of reproduction occurs through hypothalamic release of GnRH. Silvia, et al. [43] observed no differences in hypothalamic content of GnRH or the concentration of GnRH receptors in the anterior pituitary of mares during winter anovulation, the spring transitional period and breeding season. However, concentrations of pituitary LH increased markedly between the non-breeding and breeding seasons. However, Hart [18] found a correlation between hypothalamic content

of GnRH between seasons with content being lowest during non-breeding season but not anterior pituitary receptors for GnRH during the breeding season, non-breeding season, vernal transition and late breeding season. Similarly, Sharp [44] observed an increase in GnRH secretion rate in cerebrospinal fluid using a push-pull perfusion technique from the non-breeding season to the breeding season. Due to the unique cranial vasculature of the horse, early work by Irvine, Hunn [45] and Irvine, Alexander [46] made it possible to measure GnRH and gonadotropins directly from pituitary venous effluent. The technique allowed for the cannulation of the intercavernous sinus (ICS) through the superficial facial vein. In the mare, episodic secretion patterns of LH are not measurable in the peripheral circulation except during the mid-luteal phase. Moreover, similar to other mammalian species, secretion of GnRH typically must be measured either in hypophyseal portal blood or third ventricle CSF. In the mare, some investigators have been able to successfully measure episodic release of LH and GnRH [47]. A high correlation between GnRH and LH pulses were reported using the ICS technique [15,32,47,48]. Irvine, Alexander [49] reported that 90% of LH peaks were concurrent with GnRH peaks. Our group applied this technique to evaluate possible changes in GnRH that might be indicative of seasonal regulation of gonadotropins. Surprisingly, we were unable to find any aspect of GnRH secretion that differed during the four seasons [16].

Many of the methods used to increase LH and hasten the onset to first ovulation have been shown or proposed to act through the increased frequency or amplitude of GnRH release from the hypothalamus [35,47]. Numerous trials administering

exogenous, native GnRH or GnRH agonist, demonstrate that by increasing circulating concentrations of GnRH, mean circulating concentrations of LH increase markedly and this increase is capable of initiating ovarian cyclicity in anovulatory mares [50-54].

2.6 RFamide-related peptides

In the past decade, research has identified a group of peptides in the RF-amide related peptide super family of peptides shown to play a major role in regulating the secretion of LH and controlling seasonal reproduction in avian species. In the latter, the RFRP-3 sequence has been given the name, GnIH, and its homologue may play a similar role in seasonal-breeding mammalian species and evidence suggesting participation in estradiol feedback [9,55,56]. RF amide-related peptides, RFRP-1 and RFRP-3, the mammalian homologs of avian GnIH, are shown to be highly conserved inhibitory neuropeptides among bird and mammals [57]. GnIH was first isolated from the brain of Japanese quail and shown to cause a dose dependent inhibition of LH secretion from avian pituitary cell cultures [55,58]. Since this study, variations of this neuropeptide have been identified in both birds and mammals, with GnIH-RP-1/RFRP-1, GnIH-RP-2/RFRP-2, and RFRP-3, all having affinity for the receptor, GPR-147 and to a lesser extent GPR-74 [57,59].

The small peptide, RF9, or 1-adamantanecarbonyl-RF-NH2, was discovered by Simonin et al.[60] while searching for a receptor antagonist of neuropeptide FF (NPFF). The latter is an endogenous neuropeptide shown to modulate the hyperanalgesic effects of opioids, leading to an increased sensitivity to pain due to extended exposure to

opioids. The RF9 peptide proved to have a significant affinity for NPFF receptors, NPFFR1 (GPR-147) and NPFFR2 (GPR-74) and was able to reduce the hyperanalgesic effect in rats when co-administered with heroin during a paw-pressure vocalization test [60]. Our interest in RF9 is based on its ability to stimulate or disinhibit the release of gonadotropic hormones.

Since the identification of GPR-147 as the RFRP receptor and the discovery of RF9, a number of investigations have sought to determine the mechanisms behind their ability to modulate hypothalamic and pituitary secretions. The common theme among much of the research investigating RF9 is that it has been shown to reliably increase in vivo secretion of LH across various species, including mice [61], rats and hamsters [62], sheep [63], and horses [64] following either peripheral and/or intracerebroventricular (ICV) injection. Although responses are consistent, the mechanism underlying the effect of RF9 has not been definitively elucidated. While it is assumed that RF9 stimulates release of hypothalamic GnRH, direct pituitary effects have not been ruled out. Evaluation of the effects of RF9 on secretion of LH from hemipituitary explants (Pineda, et al. [61] revealed no direct effect of RF9 in the absence of GnRH. Li, et al. [65] reported an increase in LH from swine anterior pituitary cell cultures exposed to RFRP-3 and GnRH, when treated with RF9. In similar studies with sheep, the apparent role of GnRH mediating the effects of RF9 was evident. In that study RF9 elicited release of LH, but when RF9 was administered in conjunction with Teverelix, a GnRH antagonist, LH release was blocked [63]. This indicates that the mechanism of action of RF9 is likely upstream of the adenohypophysis and mediated primarily at the hypothalamus

through release of GnRH. Recent neuronal investigations in mice have shown that RF9 increases the firing rate in 70% of GnRH neurons [66]. However, by using genetically modified kisspeptin receptor (Kiss1r)-null mice the group also concluded that RF9 acts directly on GnRH neurons and contrary to much of the earlier work is dependent upon Kiss1r [66]. Although the mechanisms through which RF9 elicits its effects is still unclear, evidence suggests that it is acting directly or indirectly on GnRH neurons to increase gonadotropins.

2.7 Estradiol

The gonadal steroid, estradiol, regulates seasonal changes in the secretion of LH in the ewe through both steroid-dependent and steroid-independent systems [67]. In the ewe, increased estradiol negative feedback during the non-breeding season and changes in dopaminergic tone reduce the pulse frequency in tonic secretion of LH [67-69]. As stated above, this model has been tested in the mare with inconsistent results. In the mare, estradiol may play an important role in the transition from the anovulatory season to the breeding season by stimulating the production and secretion of LH [10,11,70]. Estradiol is at basal concentrations throughout the mid-anovulatory period and transition period [70]. Prior to first ovulation, preovulatory follicles gain steroidogenic competence and circulating estradiol concentrations begin to rise followed by a similar increase in circulating LH concentrations, leading up to the LH surge and ovulation [70,71]. Sharp, et al. [10] demonstrated that chronic treatment with estradiol in ovariectomized mares for 16 d in the early transition period will increase circulating LH. Similar research in

estrus mares treated with estradiol-17β immediately before GnRH treatment proved to effectively increase LH concentration when compared to treatment of GnRH alone, suggesting a facilitative effect from estradiol [72]. However, in contrast to Sharp, et al. [10], Mumford, et al. [73] was unable to show any significant increase in circulating concentrations of LH in early transitional mares during chronic treatment with estradiol or estradiol plus a GnRH agonist but showed an increase in releasable LH in the latter when challenged with GnRH following the treatment period. Earlier research that examined the effects of chronic treatment with estradiol-17β (10 mg/d) during the breeding season reported inhibition of follicular development, while showing serum concentration of LH to be increased during the follicular phase. Treatment resulted in an extended interovulatory interval with ovulation occurring 2 to 3d following the last estradiol injection [74,75]. This data suggests that estradiol may act to increase synthesis or secretion of LH or increase LH responsiveness to GnRH treatment in the ovulatory and transitional mare, but results are not clear, leaving questions particularly in the winter anovulatory mare.

CHAPTER III

HYPOTHALAMIC-HYPOPHYSEAL RESPONSIVENESS TO A PUTATIVE NEUROPEPTIDE FF RECEPTOR ANTAGONIST, RF9 IN THE MARE DURING THE BREEDING SEASON AND MODULATION OF ITS EFFECTS BY ESTRADIOL DURING THE WINTER ANOVULATORY PERIOD

3.1 Introduction

For the majority of the mare population, the seasonal cessation of ovarian cyclicity occurs around the winter solstice and recrudescence of ovarian cyclicity resumes after the spring equinox [1]. However, the majority of equine breed registries maintain a universal birthdate of January 1 in the Northern hemisphere [1]. This incongruence has led professional horse breeders to adopt supplemental lighting programs to advance the operational breeding season in an effort to advance foaling dates as close to the established annual birthdate as possible [4,76], and thus to provide offspring a maturational advantage at least through 3 yr of age [2,3].

Physiological mechanisms associated with the photoperiodic control of seasonality in the mare have been reviewed extensively [1,5,9]. The primary gonadotropic factor associated with the seasonal occurrence and recrudescence of ovarian cyclicity is luteinizing hormone (LH), and hypothalamic signaling pathways that regulate the secretion of LH in the mare have been described [1,13,16,70]. Several of these have been examined for their potential as pharmacological targets to accelerate resumption of anterior pituitary secretion of LH during the winter anovulatory period

and thus to expedite reproductive transition [9]. The most common approach has involved the exogenous application of GnRH and GnRH agonists to directly stimulate adenohypophyseal synthesis and secretion of LH.

During the last 10 yrs., an entirely new neuroendocrine signaling pathway involving a family of RF amide-related peptides (RFRP) associated with gonadotropin secretion and seasonal breeding in avian species has been described [58,77,78]. One of these, RFRP3 has now been named gonadotropin inhibitory hormone (GnIH). Homologs of GnIH also exist in mammals, including the horse [64]. The most prominently active of these, RFRP3 has been shown to inhibit secretion of LH in several mammalian species [58,77,78]. The first evaluations of GnIH and its putative antagonist, RF9 in the horse were reported recently by our group [8]. Results from these studies, as well as those obtained from several other species, suggest that RF9 may have a prospective role as a pharmaceutical means of stimulating secretion of LH [9,63,64,78]. However, additional characterization of hypothalamic and pituitary responses to RF9 is required in order to more fully understand its biology and to appreciate its potential as a pharmacological agent, including the role of ovarian steroids in modulating hypothalamic responses to RF9. Therefore, the objectives of the studies reported here were to test the hypotheses that 1) RF9 stimulates secretion of GnRH in concert with secretion of FSH and LH, thus confirming a hypothalamic site of action, and 2) estradiol modulates hypothalamic responsiveness to RF9.

3.2 Materials and Methods

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

3.2.1 Experiment 1. Hypothalamic-adenohypophyseal responsiveness to RF9 during the mid-luteal phase of the estrous cycle

3.2.1.1 Animals

Sixteen light horse mares, predominantly American Quarter Horses, were maintained on a pasture of mixed grasses and supplemented with Coastal Bermuda grass hay as needed to maintain a body condition score of 5 to 6 (1 to 9 scale) [79] at the Texas A&M AgriLife Research Station in Beeville, TX.

3.2.1.2 Experimental procedures

Mares were assigned randomly to one of two treatment groups: 1) control (n = 8) or 2) RF9 (n = 8). The experiment was conducted during the breeding season (Aug. 6-21). All mares were examined by transrectal ultrasonography (Honda HS-feb500V, Honda Electronics Co., LTD) every 1 to 3 d for approximately 3 wk prior to the start of the study to assess ovarian status. Estrous cycles were synchronized by regression of a functional corpus luteum (CL) using prostaglandin F2-alpha (PGF2α). Following PGF2α treatment, ovarian follicular populations were evaluated at 1- to 2-d intervals until ovulation. Treatments were initiated on day 7 to 10 following ovulation. Fifteen minutes prior to treatment, all mares received Regu-Mate® (0.044 mg/kg; DPT

Laboratories, San Antonio, TX) to inhibit the occurrence of large LH pulses associated with the luteal phase. Twenty-four hours before treatment, mares were fitted with intercavernous sinus (ICS) and jugular catheters for intensive blood sampling. The internal end of the ICS catheter was placed in close proximity to pituitary effluent based on the relationship of catheter length to facial anatomy as reported previously by our group [80,81]. Mares in the treatment group received intravenous injections of RF9 (GeneCust Europe, Dudelange, Luxembourg) in saline at times 30 and 90 min using doses of 0.2 and 0.4 mg/kg BW, respectively. Doses were based on our previously reported studies [64]. Control mares were treated similarly with saline. Blood samples were collected from both the ICS (3-5 mL) and jugular catheter (10 mL), beginning 30 min before treatment and continuing at 10-min intervals for 6 h.

3.2.1.3 Intercavernous sinus catheterization

Catheterization of the intercavernous sinus (ICS) was performed following procedures described previously [46,48,80,81]. The mare was placed in a stock and sedated with detomidine HCL (Dormosedan®, 20-40 μg/kg BW; Pfizer. New York, NY). The facial vein lies parallel to and along the anterior border of the mandible. An area centered over this line and extending in all directions for approximately 2 to 2.5 cm was clipped, scrubbed and disinfected for aseptic surgery. The facial vein was detected by palpation and an area (approximately 1 cm x 1cm) overlying and surrounding the vein, but below the facial crest, was infiltrated subcutaneously with 2% lidocaine HCL (Vedco, Inc, St Joseph, MO). A 1-cm skin incision was made over the vein, and the vein

exteriorized with blunt dissection and held in the exteriorized position by placing a sterile probe or rod between it and the underling tissue. A small cut was made in the vein while occluding flow and a Tygon® catheter (0.04 i.d. x 0.07 o.d.; Norton Performance Plastics Co, Akron, OH) was inserted into the vein. A flexible stylette was maintained in the catheter, and the catheter was gently threaded up the vein to a predetermined distance based on past procedures where radiographic imaging was used to confirm the location. If resistance was felt before reaching the predetermined distance the catheter was withdrawn to a point prior to the resistance. The objective was to thread the catheter cranially to the base of the cranial cavity. Once in place, the stylette was removed and blood flow was confirmed. If the catheter did not flow it was adjusted until flow was confirmed and a heparin (10,000 IU/mL) lock was placed in the tubing. The tubing was sutured in place with synthetic sutures (Supramid®; S. Jackson, Inc., Alexandria, VA) and the skin closed and dressed with an antiseptic skin dressing. During ICS blood sampling, the mares received 30,000 IU heparin i.v. every 3 h.

3.2.1.4 Blood collection and processing

On the day of sampling, mares were heparinized using 30,000 IU of heparin i.v., loosely tied, and provided hay and water for the duration of the collection period. An additional 30,000 IU of heparin was administered 3 h after onset of sampling. Sterile saline (5 mL) was used to flush catheters after each blood sample and 5 mL saline containing 1,000 IU of heparin was used to flush catheters hourly. Jugular samples were placed in tubes containing 50 µL 5% EDTA-heparin solution (10,000 IU/mL). The ICS

collection tubes also contained 50 µL 5% EDTA-heparin solution and an additional 100 µL of a 50 mM solution of bacitracin (Sigma Chemical co., St. Louis, MO, USA) to minimize metabolism of GnRH [47]. Upon collection, the tubes were immediately placed on ice and centrifuged (562 x g) for collection of plasma every 30 min. Plasma was stored at -20°C until hormone analysis for concentrations of LH, FSH (jugular samples) and GnRH (ICS samples).

3.2.2 Experiment 2. Effects of estradiol pretreatment on hypothalamic-adenohypophyseal responsiveness to RF9 in winter anovulatory mares

3.2.2.1 Animals

Ten light horse mares, predominantly American Quarter Horses, were maintained on a pasture of mixed grasses and supplemented with Coastal Bermuda grass hay as needed to maintain a body condition score of 5 to 6 (1 to 9 scale) [79] at the Texas A&M AgriLife Research Station in Beeville, TX.

3.2.2.2 Experimental procedures

Ovulatory status was monitored using transrectal ultrasonography (Honda HS-Feb500V, Honda Electronics Co., LTD), starting 4 wk prior to the start of the study. Mares were confirmed anovulatory based on the absence of a corpus luteum (CL), and no follicle > 30mm. Only 2 of 10 mares had follicles > 20 mm at the start of the study. Mares were stratified by largest follicle and assigned randomly to one of 2 treatment

groups 1) Control-RF9 [single i.m. injection of 2 mL corn oil followed 18 h later by i.v. injection of RF9 (0.4 mg/kg BW); n = 5], or 2) Estradiol-RF9 [single i.m. injection of 2 mL corn oil containing 5 mg estradiol-17β followed 18 h later by i.v. injection of RF9 (0.4 mg/kg BW); n = 5]. Treatment initiation was staggered over 2 consecutive d, with an equal number of mares in each group divided between the 2 d. The peptide, 1-adamantanecarbonyl-RF-NH2 (RF9; > 95 % purity) was obtained from American Peptide Company, Vista, CA. Mares were fitted with a jugular catheter at the time of corn oil/estradiol treatment, approximately 18 h before intravenous RF9 administration. Blood samples were collected from the jugular catheter at 15-min intervals starting 30 min before RF9 treatment and continuing for the first 2.5 h. Sampling frequency was then reduced to 30-min intervals for the final 3 h. Jugular catheters were removed immediately after intensive sampling.

3.2.2.3 Blood collection

On the day of intensive sampling, mares were tied loosely and provided hay and water for the duration of the collection period. The catheter was flushed following each sample as described previously (*3.2.1.4*). Samples were placed in tubes containing 50 µL 5% EDTA-heparin solution (10,000 IU/ML) to assure that samples did not coagulate. Upon collection, the tubes were immediately placed on ice and centrifuged (562 x g) for collection of plasma every 30 min. Plasma was stored at -20°C until hormone analysis for concentration of LH.

3.2.3. Hormone analysis

Plasma concentrations of LH and FSH were assayed in triplicate utilizing double antibody RIAs and GnRH was assayed in duplicate utilizing a single extraction as validated previously in this laboratory [16,82]. A highly purified equine LH preparation (LH AFP-5130A) was used for both iodinated tracer and reference standards in conjunction with anti-equine LH antiserum (AFP-240580) at a dilution of 1:120,000. A highly purified equine FSH (eFSH AFP-5022B, eFSH AFP-8830D) preparation was used for the iodinated tracer and standards in conjunction with anti-eFSH antiserum (AFP-2062096) at a dilution of 1:12,500. Highly-purified hormones and antisera were provided by Dr. A.F. Parlow, National Hormone and Peptide Program (NHPP), Harbor-UCLA Research and Education Institute, Los Angeles, CA. The sensitivity, intra- and interassay coefficients of variation (CV) for Exp. 1 were 0.25 ng/mL, 7.6% and 10.0%, respectively for LH, and 0.5 ng/mL, 5.1% and 3.7%, respectively for FSH. For Exp. 2, the sensitivity, intra- and interassay-CV were 0.25 ng/mL, 9.5% and 5.6%, respectively for LH.

The GnRH assay for Exp. 1 could not be validated. Therefore, no GnRH data are presented and the hypothesis related to secretion of this specific hormone could not be tested.

3.2.4. 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 of the JMP program associated with Statistical Analysis Systems (SAS; SAS Inst., Inc., Cary, NC). The model included LH- and/or FSH-dependent variables, using day as the within subject factor, treatment as the between-subject factor, and mare as the subject. Fixed effects were treatment, day, and treatment x day interaction. In Exp 1, only mares with successful ICS cannulations were used in the trial (Control, n = 6; RF9, n = 7). In Exp 2, all mares were used in the analysis (Control-RF9, n = 5; Estadiol-RF9, n = 6). If fixed effects were found significant, a pair-wise comparison was performed using least squares means Student's t-test. Main effects were considered significant when $P \leq 0.05$ and a trend toward significance when $P \leq 0.10$

3.3 Results

3.3.1 Experiment 1. Hypothalamic-adenohypophyseal responsiveness to RF9 during the mid-luteal phase of the estrous cycle

Mean concentrations of LH and FSH did not differ among groups during the pretreatment period (-30 to 0 min.); however, within 20 min following the first injection of RF9 (0.2 mg/kg BW), circulating concentrations of LH and FSH had both increased ($P < 0.05$ and $P < 0.04$, respectively) above the control (Fig. 3.1). Circulating LH concentrations increased to a maximum mean concentration 40 min following the second bolus treatment of RF9 (0.4 mg/kg BW), with a mean peak difference from the control of 1.2 ± 0.3 ng/mL ($P < 0.003$). Follicle stimulating hormone demonstrated a similar response but reached a maximum mean concentration at 30 min following the second bolus treatment of RF9, with a mean peak difference from the control of 4.3 ± 1.4 ng/mL

($P < 0.0003$). Response curves for both LH and FSH demonstrated a similar linear decay from peak concentrations and reached pretreatment concentrations by the end of the 6-h blood sampling period. The overall treatment effect on mean concentrations of LH and FSH following the initiation of RF9 treatments were greater ($P < 0.05$ and $P < 0.02$, respectively) in RF9-treated than in control mares. A treatment x time interaction ($P < 0.001$) was noted for both hormones, with hourly means differing as depicted in Fig. 3.1.

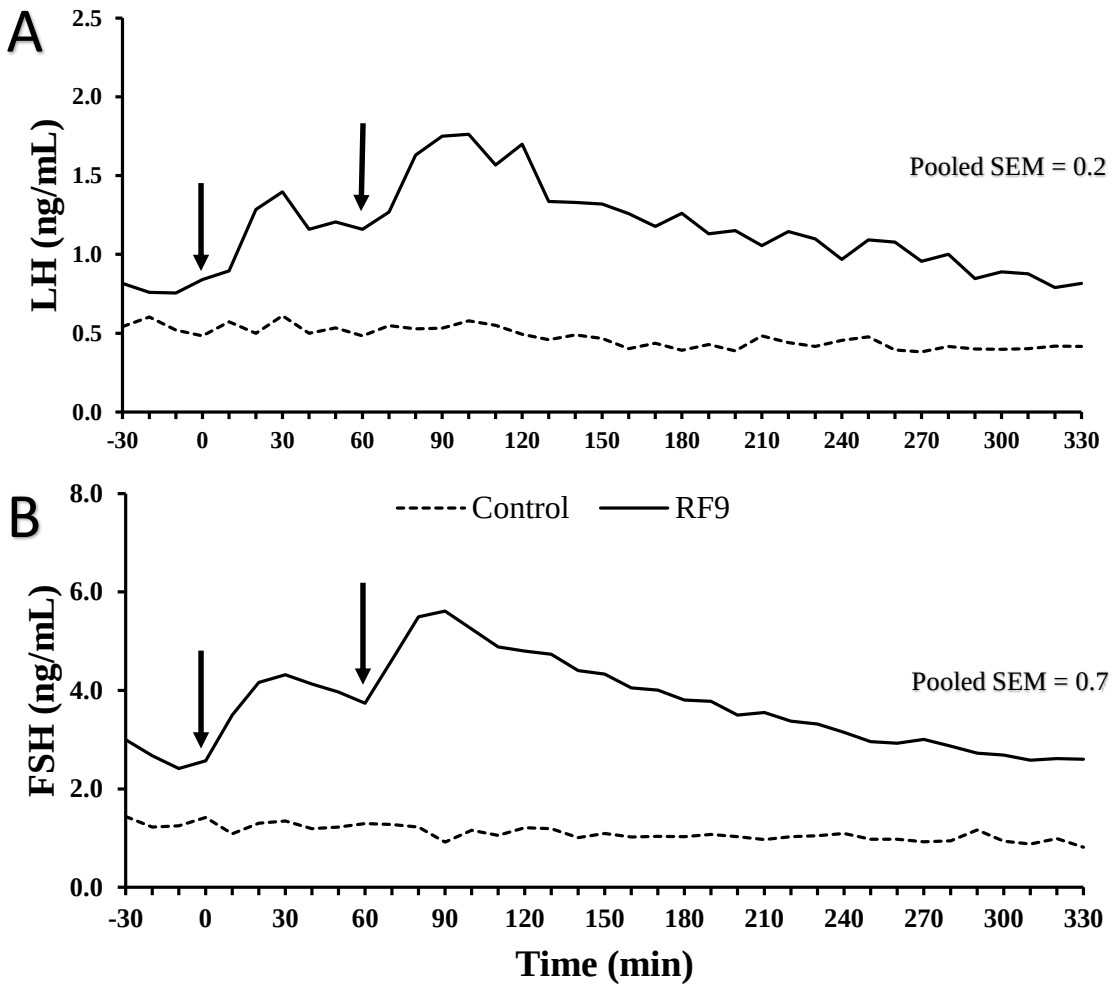

Fig. 3.1. Least squares mean concentrations of LH (A) and FSH (B) over the 6-h sampling period in Control (dashed line; n = 6) and RF9-treated (solid line; n = 7) mares. Arrows denote RF9 or saline treatments at time 0 (RF9 = 0.2 mg/kg BW) and 60 (RF9 = 0.4 mg/kg BW) min, respectively. LH and FSH Treatment x time $P < 0.0001$.

3.3.2 Effect of estradiol pretreatment on hypothalamic-pituitary responsiveness to RF9 in winter anovulatory mares

Mean concentrations of LH did not differ between groups during the pretreatment period (-30 to 0 min). Concentrations of LH in both Control-RF9 and Estradiol-RF9 increased ($P < 0.02$; $P < 0.0001$ respectively) following RF9 treatment,

with maximum concentrations of LH observed at 30 min following RF9 injection (Fig. 3.2.). Although the overall mean concentrations of LH did not differ for the 5-h post-treatment period, there was a treatment x time interaction ($P < 0.04$). The mean peak concentration of LH occurring at 30 min post-treatment was greater ($P < 0.05$) in Estradiol-RF9 treated than in Control-RF9 treated mares.

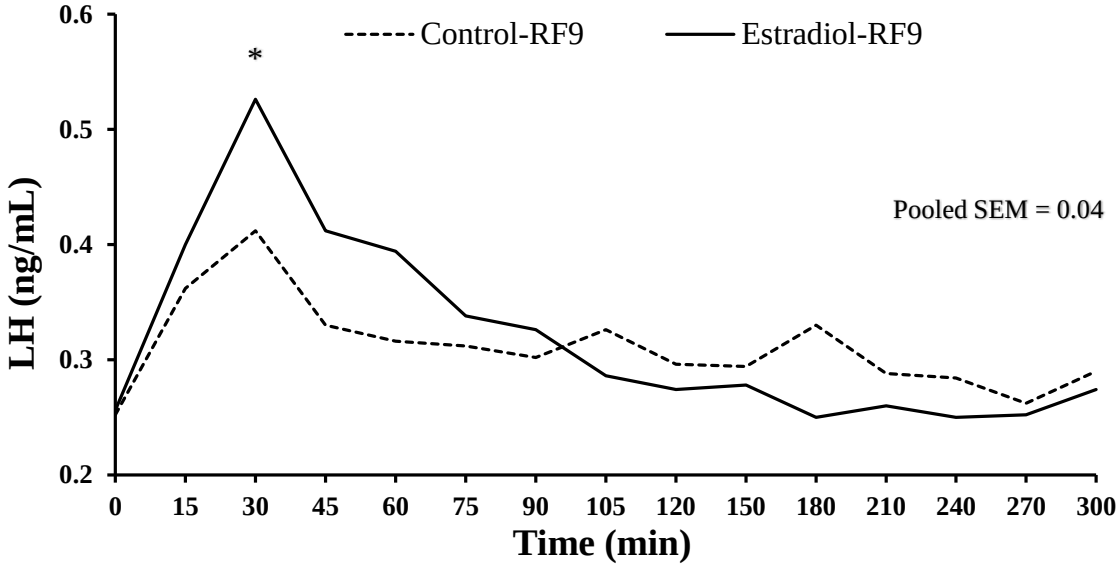

Fig. 3.2. Least squares mean concentrations of LH in the Control-RF9 (dashed line) and Estradiol-RF9 (solid line)-treated mares. Mares were treated at time 0 min and mean concentrations of LH did not differ during the pretreatment period (-30-0 min). Differences are indicated by (*) $P < 0.05$. Treatment x time $P < 0.04$

3.4 Discussion

Confirmation that RF9 stimulates the secretion of GnRH from the hypothalamus, based on in vivo experiments, has not been reported. One of the objectives of studies reported herein was to address this question using the ICS cannulation technique in the mare. This approach facilitates measurement of pituitary, and potentially, hypothalamic

hormone secretion by sampling pituitary venous effluent. Unfortunately, this objective was not met in the current work due to the inability to validate measurement of GnRH in ICS plasma. However, the blockade of RF9 mediated release of gonadotropins by GnRH antagonists suggests a GnRH-dependent effect in the ewe and the mouse [62,63]. Moreover, the putative RF9 receptor, GPR147, has been identified on GnRH and kisspeptin neurons in the rostral paraventricular region of the hypothalamus. In contrast, recent investigations using the Kiss1receptor (Kiss1r)-null mouse suggest that RF9 is acting on GnRH neurons through activation of Kiss1r [66]. These and similar observations have led to increasing recognition of RF-amide related ligands cross reacting with several receptors [83].

Results of the current experiments confirm the ability of RF9 to reliably increase circulating concentrations of LH in the mare during both the winter anovulatory period and natural breeding season. We also show for the first time the ability of RF9 to stimulate secretion of FSH in the mare in a manner that mirrors that of LH, with no indication of differential responsiveness. Earlier reports in the ewe have shown a similar response [63]. While Exp. 1 and 2 were not designed to compare seasonal responses, it is obvious that RF9-mediated secretion of LH is greater during the breeding season compared to the non-breeding season. This is readily explained in the mare by the fact that pituitary content of LH during the winter anovulatory period is markedly depleted relative to the breeding season [18,84]. Thus, it is expected that the ability of the anterior pituitary to release LH in response to any secretagogue would be compromised in the mare during the non-breeding season. However, this is in contrast to observations in the

seasonal breeding ewe, where the lesser response is observed during the breeding season. Pituitary content of LH is not depleted during the anestrous season in ewes. Thus, pituitary content and RF9 responsiveness are lower during the breeding season of the ewe due to greater depletion of LH coincident with a greater rate of endogenous secretion [63].

The gonadotropic response to RF9 has been well established by our group and others across several mammalian species including the horse, rodents, and sheep [61-64]. In the current study, we have expanded this information by examining the ability of the ovarian steroid hormone, estradiol-17β to modulate RF9 responsiveness during winter anovulation. Results indicate that estradiol pre-treatment enhanced the response to RF9, with mean LH peak greater following treatment with RF9. The effect of estradiol in the mare during the transition from winter anovulation to first annual ovulation has not been well characterized. However there is evidence that LH synthesis is increased in ovariectomized mares during vernal transition [11]. In seasonal breeding sheep, estradiol treatment has been shown to increase the number of GnRH receptors on gonadotropes [85]. Although not examined directly in the mare in response to estradiol, concentrations of anterior pituitary GnRH receptors were reported to be unchanged during different stages of the estrous cycle [18]. However, a decrease in pituitary content of GnRH receptors has been reported in the mare during the seasonal anovulatory period compared to the breeding season [43]. If estradiol is upregulating the GnRH receptor in the mare, and RF9 stimulates GnRH release, this may explain why a similarly-enhanced response is seen with direct treatment with GnRH [86].

Estradiol has been reported to downregulate the expression of RFRP in the mouse, suggesting a decrease in inhibition of GnRH neurons [56]. This could account for the ability of estradiol to enhance RF9's stimulatory effects in the current work. Although there remains uncertainty regarding the mechanism of RF9's primary action through GPR147, RFRP3's affinity to the receptor has been supported [66,87]. In fact, in the GPR147-null mouse, RFRP3 was unable to suppress serum concentrations of LH [87]. Although our group has been unable to demonstrate in vivo or in vitro (via pituitary cell culture) inhibition of LH secretion in the mare following treatment with RFRP3, we have confirmed the RFRP3 machinery is present in the mare, including presence of hypothalamic neurons immunoreactive for RFRP3 and anterior pituitary expression of the NPFFR receptor [64,88]. Also, as indicated earlier, RF9 can act through direct activation of GPR54. Thus, estradiol could exert its affects through increased expression of GPR54 in GnRH cells as seen in cultured GT1-7 GnRH cells following 24 h exposure to estradiol.

In conclusion, the results from the current experiments confirm the ability of RF9, a putative RFRP-3 receptor antagonist, to increase circulating concentrations of LH during both the breeding and non-breeding seasons of the mare in concert with a similar ability to stimulate release of FSH. These and other related reports suggest that RF9 creates these effects by stimulating the release of GnRH from the hypothalamus. However, we were not able to confirm this in the current work. We have also demonstrated that estradiol may enhance responsiveness to RF9 but the location of action of this effect remains to be determined.

CHAPTER IV

DEVELOPMENT OF A LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY PROTOCOL TO QUANTIFY METABOLIC CLEARANCE RATE OF EXOGENOUSLY-ADMINISTERED RF9 IN THE MARE

4.1 Introduction

Previous studies have shown that low peripheral plasma concentrations of LH serve as the primary basis of ovarian quiescence during the non-breeding season in the mare [13]. Low circulating LH occurs as a consequence of reduced secretion of GnRH and appropriate treatment of seasonally anovulatory mares with GnRH increases LH and can restore ovulatory cycles [53,89]. A potential role for RF amide-related peptides in regulating seasonality in horses has been proposed to cause a decrease in GnRH release that is accompanied by a decrease in downstream release of LH [9]. Similarly, RF9, a putative RFRP receptor signaling antagonist, has been shown to stimulate the release of LH in several mammalian species, including the winter anovulatory mare [8,61,63]. It is of interest, therefore, to characterize the pharmacological potential of RF9 in this setting. The objectives of this experiment were to develop a means of quantifying RF9 using liquid chromatography/mass spectrometry (LC/MS) and to use this approach for assessing the rate of disappearance of exogenously-administered RF9 in the mare.

4.2 Materials and Methods

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

4.2.1 Experimental procedures

The plasma used for the blanks were from a single mare that had not been exposed to RF9 and was stored at -20°C. Plasma samples used for this assay were from previous work conducted by Thorson, et al. [8]; 5 RF9-treated mares and 3 Controls or non-treated mares. Blood samples obtained in Thorson's research were collected over 6 h and drawn from an indwelling intravenous jugular catheter at 10-min intervals (Fig. 4.1.). The RF9 was administered at a dose of 0.4 mg/kg body weight at 60 min and 120 min after intensive sampling was initiated to evaluate secretion of LH from the anterior pituitary. Samples were placed into tubes containing 50 µL ethylenediaminetetraacetic acid-heparin solution to prevent coagulation and placed directly on ice. Every hour post-harvest, samples were processed by centrifugation for plasma collection and stored at -20°C until thawed for determination of concentrations of LH by RIA. Following the LH RIA assay, plasma was again stored at -20°C until briefly thawed for LC/MS evaluation of RF9 concentrations in peripheral circulation. For the purposes of our assay, we chose samples prior to treatment (t = 50 min) and samples immediately following treatment (t = 70, 130 min). To evaluate its half-life, we chose samples 140 min and 240 min following the last treatment based on earlier evaluation in sheep reporting a 2.1 h

terminal half-life [63]. The non-treated mares (negative controls) were used to evaluate if there are biological factors in regular plasma that would lead to a false positive.

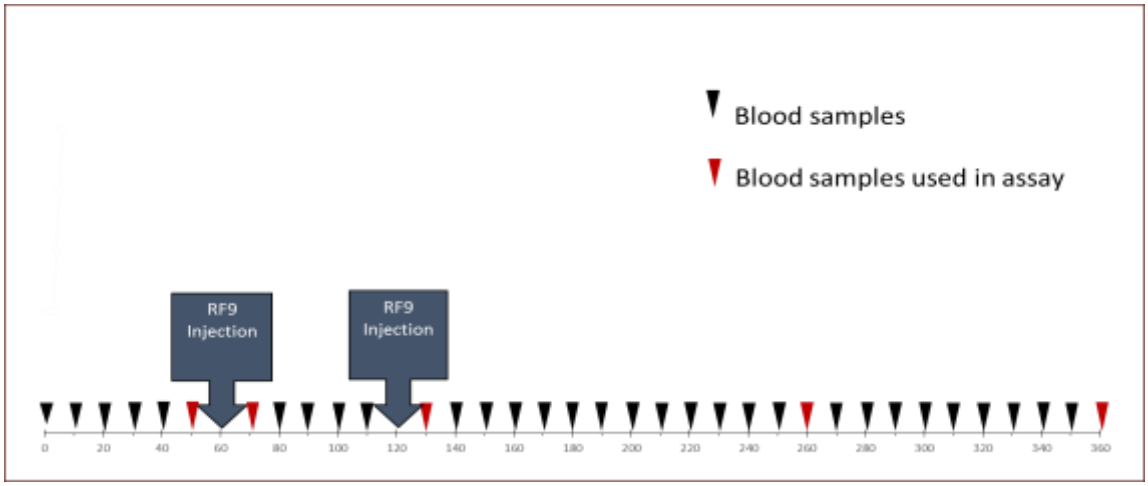

Fig. 4.1. Time line of sampling with red arrow heads indicate time points used in the current study.

4.2.2 Preparation of standards

The stock solution was first prepared by dissolving 1 mg of RF9 in 1 mL methanol. Then the concentrated methanol was diluted 500 fold to make 5 mL of stock solution at 2 µg/mL. The stock solution was then stored at -20° C and thawed prior to preparation of calibration standards and stored in ice while dilutions were being made.

The internal standard (I.S.), testosterone-D3 (16, 16, 17-D_3) in stock solution was at 100 µg/mL in acetonitrile and stored at -20°C. Testosterone –D3 was added to water, acetonitrile, and formic acid (89.9:10:0.1 v/v) to make the resuspension solution daily at 100 ng/mL and volume varied based on number of samples being analyzed that day.

Calibration standards were made daily by first spiking 190 µL of PBS with 10 µL of the stock solution to create 200 µL of working solution (100 ng/mL). Dilutions

were made by spiking 100mL of PBS with the working solution to the desired concentrations 2, 5, 10, 20, 50, 80, 100 ng/mL and the 300, 500, 1000, 2000 ng/mL standards made by spiking 100 µL PBS with the stock solution. Then 100 µL of blank plasma was added to each vial, vortexed for 30 s, and allowed to equilibrate for 2 min before extraction. The calibration standards were then extracted with the analysis for that day using the following extraction method.

4.2.3 Sample extraction

One-hundred microliter aliquots of plasma from 8 mares (non-treated, n = 3; RF9 treated, n = 5) or calibration standard in blank plasma was added to a 1.5-mL Eppendorf-style tube and diluted with 100 µL of PBS, mixed for 30 s and allowed to sit on ice for 2 min. Five-hundred microliters of acetonitrile with 0.1% formic acid was added to each tube, mixed for 30 s and allowed to sit on ice for an additional 2 min before centrifugation at 9447 x g for 15 min. After centrifugation, 400 mL of the aqueous supernatant fluids were transferred into a clean 1.5-mL Eppendorf-style tube and dried using vacuum centrifugation (SpeedVac). An additional 40 uL of the extract supernatant was removed for the time 70 and 130 samples to create 10-fold dilutions, ensuring they would fall within the measurable range of the assay. The dried samples were then dissolved in 100 µL of reconstitution solution containing the internal standard, mixed for 30 s and for an additional 10 min in a sonicated water bath. The tubes was then centrifuged again at 9447 x g for 10 min and 50 µL of the supernatant fluids were transferred to an autosampler vial, taking care not to disturb the precipitant pellet. The

samples were loaded into a chilled drawer of the Accela auto-sampler cabinet. An aliquot of 20 µL was injected into the LC/MS system for analysis.

4.2.4 Chromatographic conditions

All chromatography was performed on an Acclaim Polar II C18 column (3 µm, 2.1 x 50 mm, ThermoScientific) operating at room temperature. Both RF9 and testosterone-D3 were tuned separately for optimum sensitivity. The method was then developed to incorporate the optimal chromatographic conditions for both substrates.

4.2.5 Mass spectrometry conditions

Mass spectrometry was performed by use of an Exactive Orbitrap mass spectrometer (ThermoScientific, San Jose, CA) equipped with a heated electrospray ionization source operating in positive mode. Nitrogen was used as sheath, auxiliary and sweep gases. Different parameters were used for RF9 and Testosterone-D3 to optimize sensitivity for each substance. Method development and data acquisition was performed with Trace Finder v3.1 (ThermoScientific).

4.2.6 Pharmakinetic analysis

The means of the 5 treated mares following the last RF9 treatment (t = 130, 260, 360) were evaluated using PKSolver 2.0, a pharmakinetic and pharmacodynamics data analysis add-in for Microsoft Excel [90]. The analysis was done using the non-compartmental approach for plasma after intravenous bolus input.

4.3 Results

The quantitative recovery of RF9 from equine plasma was demonstrated and by utilizing Trace Finder v3.1 (ThermoScientific) we also demonstrated a standard calibration curve for RF9 that was linear (r2 = 0.999) over the concentration range of the assay (2 to 2000 ng/mL) using a weighted least square linear regression analysis with a weighing factor of 1/X (Fig. 4.2.). The representative regression equation for the calibration curve was $y = 8.158e\text{-}8x^2 + 5.29e\text{-}3$. The lower limit of detection for the assay was 2 ng/mL, with the lower limit of quantification designated at 20 ng/mL. The intra-assay CV of the assay determining RF9 concentrations in the mares evaluated was 3.8%. The negative controls did present low levels of detection with a maximum range from 0 to 9.3 ng/mL with a mean (± SEM) of 2.0 ± .6 ng/mL. Analysis of RF9 circulating concentrations in the mare proved to have a rapid increase following treatment and also expressed a rapid decline following treatment (Fig. 4.3.) with a predicted clearance rate (CL) of 0.000912 mg/(ng/mL)/min and half-life (t1/2) 40 min calculated from the mean CL of RF9 from the five treated mares (Fig. 4.4.).

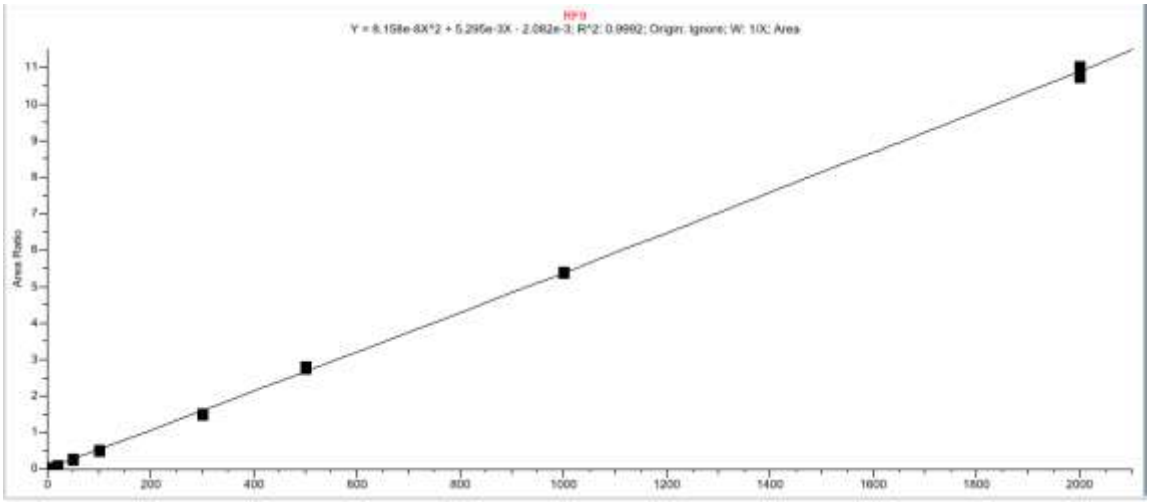

Fig. 4.2. Linearity was determined to assess the performance of the method. A least-squares regression fit to a quadratic equation with a weighting index of 1/X order was performed on the peak area ratios of RF9 and I.S. versus RF9 concentrations of the ten plasma standards (2, 5, 10, 20, 50, 100, 300, 500, 1000 and 2000 ng/mL) in duplicate to generate a calibration curve.

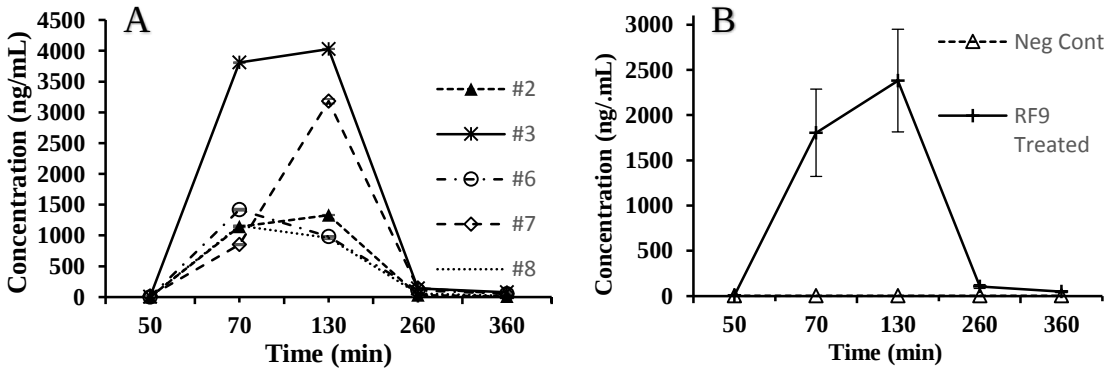

Fig. 4.3. RF9 in equine plasma. A) RF9 in five treated mares. B) Average of the five treated mares including the average of the three non-treated mares. Error bars represent ±SEM

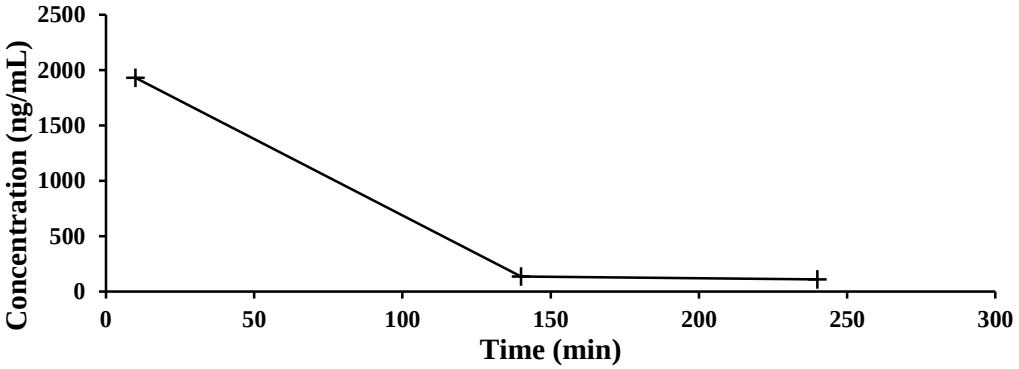

Fig. 4.4. Half-life was calculated using the mean plasma concentrations of last three time points. PKSolver was used in a non-compartmental analysis of plasma following bolus i.v. infusion. (t1/2 = 40 min)

4.4 Discussion

The results from previous work by our group has proven RF9 to be a reliable means of pharmacologically increasing circulating concentrations of LH in cyclic and acyclic mares [8,64] (Korthanke unpublished). Because of RF9's reliability to increase LH, a pharmacokinetic evaluation of its half-life in the circulatory system of the mare was warranted to allow for further investigation of its practical application as a pharmacological agent through various dose regiments. Results indicate that we were able to successfully extract RF9 from equine plasma and develop an accurate means to quantify its presence in plasma using an LC/MS method that allowed further evaluation of its elimination from the blood over time. Using the cumulative average of all the mares, we showed that within 4 h RF9 has been eliminated from the circulation to a point near the LLOQ for our assay. Using the PKSolver application provided by Microsoft Excel, we were able to determine the t1/2 and predicted CL of RF9 in the mare for further dosage calculations.

Because of the dose required to obtain a significant gonadotropic response to RF9 in an animal as large as the horse, and metabolic clearance data presented here, it appears unlikely that RF9 could be used chronically to stimulate secretion of gonadotropins in the equine species under practical conditions. Employing methods used previously by our group (e.g., Alzet Osmotic Pumps; DURECT Corp., Cupertino, CA) and the solubility of RF9, it appears it would be impossible to program effective delivery over extended periods of time because of the small maximum volume and flow rate of these pumps.

In conclusion, we were able to develop a method to extract and quantify RF9 from equine plasma for further pharmacological investigations. Results indicated that the pharmacological application of RF9 may be limited in horses to acute treatment because of its short half-life and limitations in availability of delivery platforms for continuous applications.

CHAPTER V

ESTRADIOL AND ITS INTERACTION WITH GnRH IN REGULATING SECRETION OF LH IN WINTER ANOVULATORY MARES DURING INCREASING PHOTOPERIOD

5.1 Introduction

The mare is a seasonal long-day breeder with approximately 85% becoming anovulatory by the time of the winter solstice. Seasonal recrudescence of ovarian cyclicity occurs after the spring equinox coincident with increasing day length [2]. The onset of the seasonal anovulatory period is characterized by a marked decline in circulating concentrations of LH that precedes the arrest of ovulatory cycles. Similarly, the resumption of reproductive cyclicity occurs as a consequence of a marked increase in synthesis and secretion of LH. Reproductive periodicity in seasonal species is synchronized by environmental cues and, in the mare, day-length is the primary environmental factor influencing reproductive patterns. While many of the physiological mechanisms underlying seasonal reproduction have been characterized, a full understanding of these mechanisms, particularly in the horse, remains lacking [9].

Vernal transition usually begins during increasing photoperiod around the time of the spring equinox and includes increased secretion of LH and ovarian follicular development. Associated with this activity is an increase in estradiol secretion as follicles gain steroidogenic competency [70,71]. Some previous reports indicate that estradiol is stimulatory to the reproductive neuroendocrine system in the mare and

increases secretion of LH [10,11,91]. Notable examples include the occurrence of foal heat in mares foaling during the winter [92,93] and in response to treatment with estradiol during increasing photoperiod [10,11]. However, reports on changes in secretion of LH in response to exogenous estradiol during increasing photoperiod have been contradictory [10,73]. Sharp et al. [10] observed an increase in LH during a 16-d treatment period with estradiol-17β in ovariectomized mares during vernal transition. Similarly, experiments conducted over the interovulatory period in the early (May and June) and late (August and September) breeding season have shown an increase in concentrations of LH following regression of the corpus luteum (CL) [72,74]. However, Mumford et al.[73] was unable to show any increase in LH over an 8-d treatment period or an increase in releasable LH following a GnRH agonist challenge. Similarly, estradiol for 4 d followed by GnRH + estradiol for an additional 4 d was unable to increase circulating concentrations of LH but, when challenged on day 10, an increase in releasable LH was observed [73]. At the cellular level, chronic treatment with estradiol has been shown to enhance LH biosynthesis in the vernal transitional mare [11]. In ovine pituitary cell cultures, estradiol increases the concentration of GnRH receptors but this has not been examined in the equine. Therefore, uncertainty remains regarding the influence of estradiol on the hypothalamic-pituitary axis of the mare and its potential interaction with GnRH during the anovulatory period.

Objectives of the study reported here were to test the hypothesis that chronic treatment with estradiol, with or without simultaneous treatment with GnRH, will

increase circulating concentrations of LH and stimulate ovarian follicular growth in winter anovulatory mares during increasing photoperiod.

5.2 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.

5.2.1 Animals

Ten barren light horse mares, predominantly American Quarter Horses, were maintained on a pasture of mixed grasses and supplemented with Coastal Bermuda grass hay as needed to maintain a body condition score of 5 to 6 (1 to 9 scale) [79] at the Texas A&M AgriLife Research Station in Beeville, TX.

5.2.2 Experimental procedures

Ovulatory status was monitored using transrectal ultrasonography (Honda HS-Feb500V, Honda Electronics Co., LTD), starting the second week in January and continued for 4 wk prior to the start of the study. Mares were confirmed anovulatory based on the absence of a CL and no follicle > 30mm during this time period. Mares were stratified by largest follicle size and assigned randomly to one of 4 treatment groups: 1) Control [Sham pump and twice-daily i.m. injections of 2 mL corn oil; n = 5], 2) GnRH [Alzet osmotic pump providing continuous subcutaneous delivery of GnRH (100 µg/h) and twice daily i.m. injections of 2 mL corn oil; n = 5], 3) Estradiol [sham

pump and twice daily i.m. injections of 5 mg estradiol in 2 mL corn oil; n = 5], 4) GnRH + Estradiol [combination of treatments 2 and 3; n = 5]. The experiment was conducted during the non-breeding season and initiated in each treatment group over 2 consecutive days (February 12 and 13). An Alzet osmotic minipump (model 2ML2; DURECT Corp., Cupertino, CA) or sham pump constructed of Silastic tubing (Dow Corning Corporation, Midland, MI) filled with medical grade silicone adhesive (Dow Corning Corporation, Midland, MI) was implanted surgically under the skin on the first day of the experiment (day 0) and removed on day 14. Twice daily treatments with estradiol-17β or corn oil were begun on day 0 and continued until confirmation of ovulation or 14 d, whichever occurred first. Ovarian follicular populations were evaluated every 1 to 3 d using transrectal ultrasonography (Honda HS-Feb500V, Honda Electronics Co., LTD) beginning before pump insertion and ending on day 14 or following ovulation, whichever occurred first. Estrous behavior was determined daily in a large pen of 4 to 5 mares using an intact stallion in an adjacent pen separated by a solid panel, with teasing scores assessed on a scale of 1 to 4 [53].

5.2.3 Subcutaneous pump insertion

The mare was placed in a stock and sedated with detomidine HCL (Dormosedan®, Florham Park, New Jersey, 20-40 µg/kg BW if required. An area at the base of the neck was clipped and prepared of aseptic surgery using an iodophore (Purdue Product LP, Stamford, CT), providone iodine solution (Prepodyne, WestArgo, Kansas City, 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, creating a pocket to accommodate the osmotic or sham pump. The incision was closed with #2 non-absorbable synthetic sutures (Supramid®) that was left in place for 7 d. The wound was dressed with a wound dressing for horses. On the last day of the experiment, the incision was re-opened and the pump was removed. The incision was closed again with #2 non-absorbable synthetic suture (Supramid®) and sutures were removed after 7 d.

5.2.4 Blood sampling

Single blood samples were collected daily via jugular venipuncture starting immediately before pump insertion and placed into sterile 10-mL blood collection tubes containing 15.0 mg EDTA. Samples were taken at approximately the same time every morning before the mares were treated and samples placed on ice or under refrigeration (4°C). Samples were centrifuged (562 x g) for 30 min at 4°C for the collection of plasma. Plasma samples were stored at -20°C until hormone analysis for concentrations of LH.

5.2.5 Hormone analysis

Plasma concentrations of LH were assayed in triplicate utilizing a double antibody RIA as validated previously in this laboratory [82]. 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 1:120,000. Highly-purified hormones and antisera were provided by Dr. A.F. Parlow, National Hormone and Peptide Program (NHPP), Harbor-UCLA Research and Education Institute, Los Angeles, CA. The sensitivity, intra- and interassay coefficients of variation were 0.25 ng/mL, 11.1% and 10.3%, respectively for LH.

5.2.6 Ultrasonography

Transrectal ultrasonography was performed every 1 to 3 d beginning prior to pump insertion. When a follicle ≥ 30 mm in diameter was detected, ultrasound examinations were performed daily until ovulation was confirmed. Upon conformation of ovulation, no further evaluations were performed.

5.2.7 Estrous behavior

Mares were teased daily in a large pen of 5-6 mares using an intact stallion in an adjacent pen separated by a solid panel. Teasing scores were assessed utilizing 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 aggression evidenced by kicking, and ear-pinning) [53].

5.2.8 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 of an add-in of JMP from the Statistical Analysis Systems (SAS; SAS Inst., Inc., Cary, NC)). The model included day or period as the within subject factor, treatment as the between-subject factor, and mare as the subject. Fixed effects were treatment, day or period, and treatment x day or treatment x period interaction. If fixed effects were found significant, a pair wise comparison was done using means Tukey's HSD. Main effects were considered significant when $P \leq 0.05$ and a trend toward significance when $P \leq 0.10$.

5.3 Results

5.3.1 Plasma LH

Two mares from the GnRH treatment group ovulated on days 8 and 9, respectively and 1 mare from the GnRH + Estradiol (GnRH/E2) treatment group ovulated on day 9. Therefore, the analysis of mean concentrations of LH among groups included values only up to the time of ovulation for those mares. Also, post-treatment concentrations of LH in mare 44 were 2 standard deviations above the mean of the treatment group. Therefore, mare 44 was removed from consideration (Fig. 5.1.).

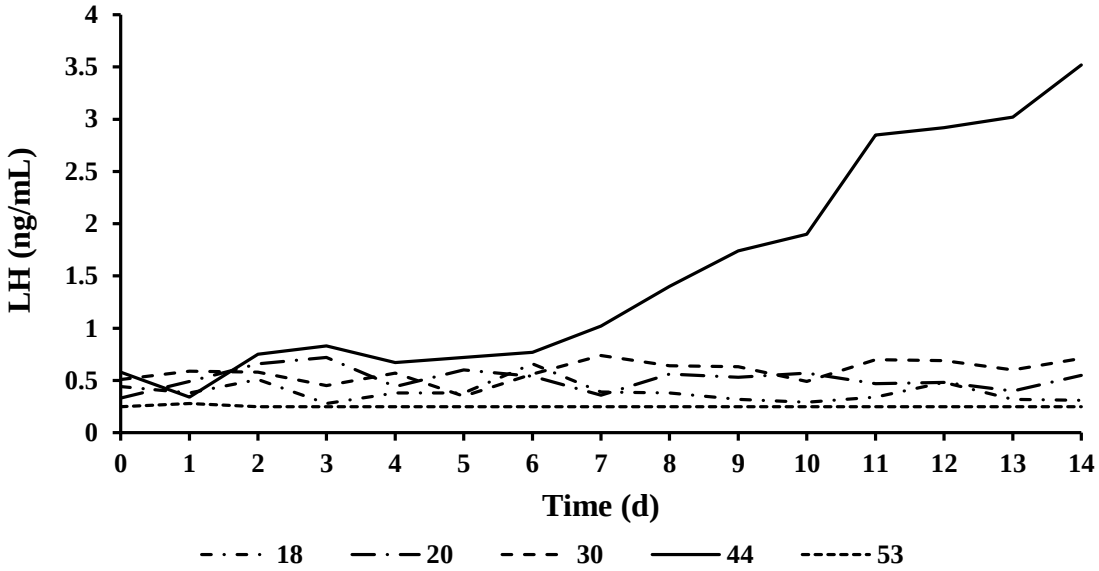

Fig. 5.1. Individual concentrations of LH for mares in the estradiol-treated group. Mare 44 exhibited an increase in circulating concentrations of LH, with a concentration (±SED) on day 14, 3.1 ± .1 ng/mL above the other 4 mares.

Mean circulating concentrations of LH did not differ ($P = 0.355$) among treatment groups on day 0 (Fig. 5.2.). The main effects of treatment ($P < 0.0001$), and the treatment x day interaction ($P < 0.0001$) on peripherally circulating concentrations of LH were highly-significant. Post-treatment mean concentrations of LH were greater in mares treated with GnRH/E2 ($P < 0.0002$) and GnRH ($P < 0.009$) than in control mares.

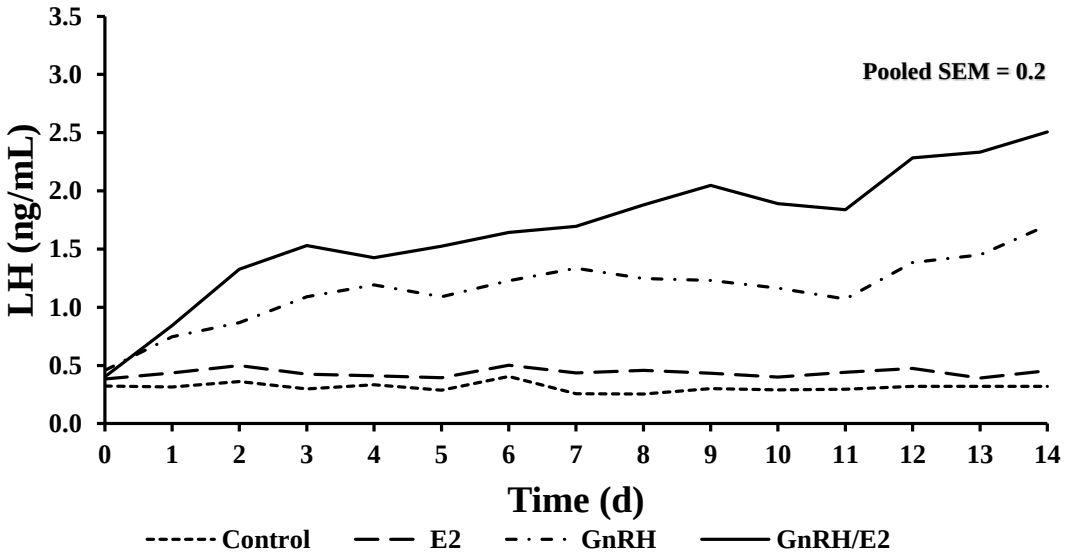

Fig. 5.2. Least squares mean concentrations of LH over the 14-d treatment period, with all treatments beginning on day 0. Overall pooled mean concentrations of LH were greater for GnRH/E2 ($P < 0.0002$) and GnRH ($P < 0.009$) compared to the controls. Treatment x day interaction $P < 0.0001$.

Because of the significant treatment x time interaction, a post-hoc analysis was performed in which concentrations of LH were pooled over 3-d periods to form 5 periods (I - V). Fig. 5.3 depicts pooled mean concentrations of LH over these 3-d periods. During Period I, concentrations of LH for both GnRH ($P > 0.008$) and GnRH/E2 ($P < 0.001$) were greater than the control. During the second period, GnRH/E2 increased circulating LH concentrations above all other treatment groups ($P < 0.02$) and continued to do so for the remaining periods. Concentrations of LH in the GnRH treatment group were also greater than the E2 and Control groups from the second period ($P < 0.0001$) through the remaining periods. During the second through the fifth periods, concentrations of LH in the GnRH/E2 group were also greater ($P < 0.02$ to $P < 0.004$) than mares treated with GnRH only.

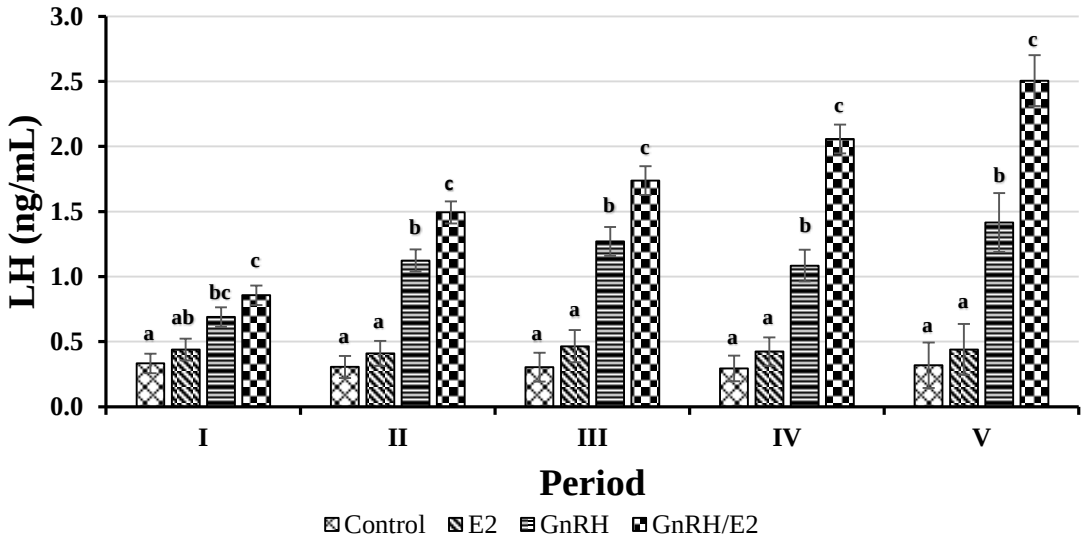

Fig. 5.3. Mean concentrations of LH pooled over 3-d periods within groups. Letters above bars (a,b and c,d) denote significant differences ($P < 0.05$) within periods. Estradiol treatments are denoted as E2 and GnRH/E2, respectively. Roman numeral I to V denote the five 3-d periods.

5.3.2 Ovarian dynamics

Three mares ovulated during the treatment period, 2 mares from the GnRH group (day 8 and 9) and 1 from the GnRH/E2 group (day 9). Mean maximum follicle size did not differ among treatment groups at the beginning of the experiment ($P = 0.38$; Fig. 5.4A) or in response to any treatments, including those groups in which mares ovulated by day 14 (Fig. 5.4B). However, mares receiving estradiol or estradiol + GnRH had numerically fewer follicles ≥ 30 mm than the GnRH group (Table. 5.1.) but a larger number of mares would be required to confirm statistically relevant effects.

Table 5.1. Number of mares from each group that ovulated and the number of mares that had follicles > 30 mm.

Treatment	Ovulations	Mares with follicle ≥ 30 mm
Control	0 of 5	1 of 5
Estradiol	0 of 5	0 of 5
GnRH	2 of 5	3 of 5
GnRH/E2	1 of 5	1 of 5

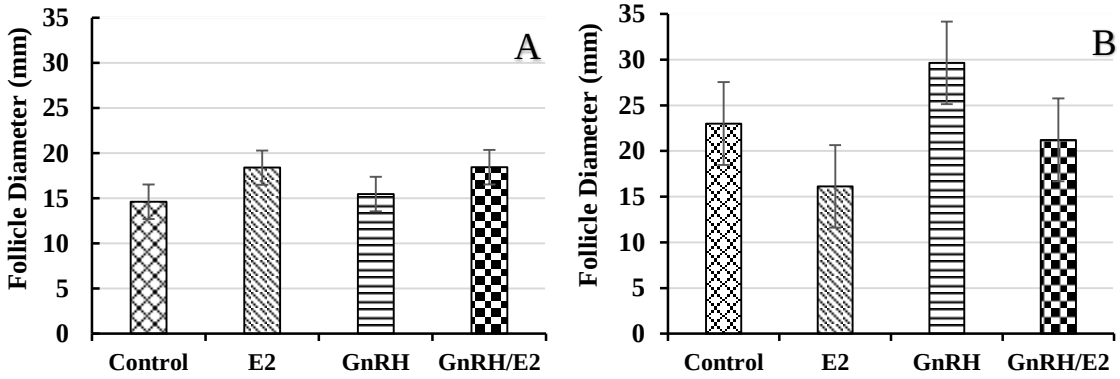

Fig. 5.4. Mean size of the largest follicle on day 0 (A) and on day 14 or the day before ovulation in 2 GnRH and 1 GnRH/Estradiol mares that ovulated (B). Mean maximal follicle size did not differ among groups.

5.3.3 Estrous behavior

Teasing scores for mares in the GnRH, Estradiol, and GnRH/E2 groups did not differ; however, all 3 of these groups had lower ($P < 0.03$) mean teasing scores in comparison to the control (Fig. 5.5.). Mean teasing score within the Control group did decline ($P < 0.01$) over the 14-day period from a mean of 3.3 ± 0.5 on day 2 to 1.2 ± 0.4 on day 14. Mean duration of consecutive days of estrous behavior (teasing score of 1) were 8.4 ± 1.7 d, 6.8 ± 0.9 d, 6.4 ± 2.2 d, and 4 ± 0.5 d for Estradiol, GnRH, GnRH/E2, and Control and did not differ among groups.

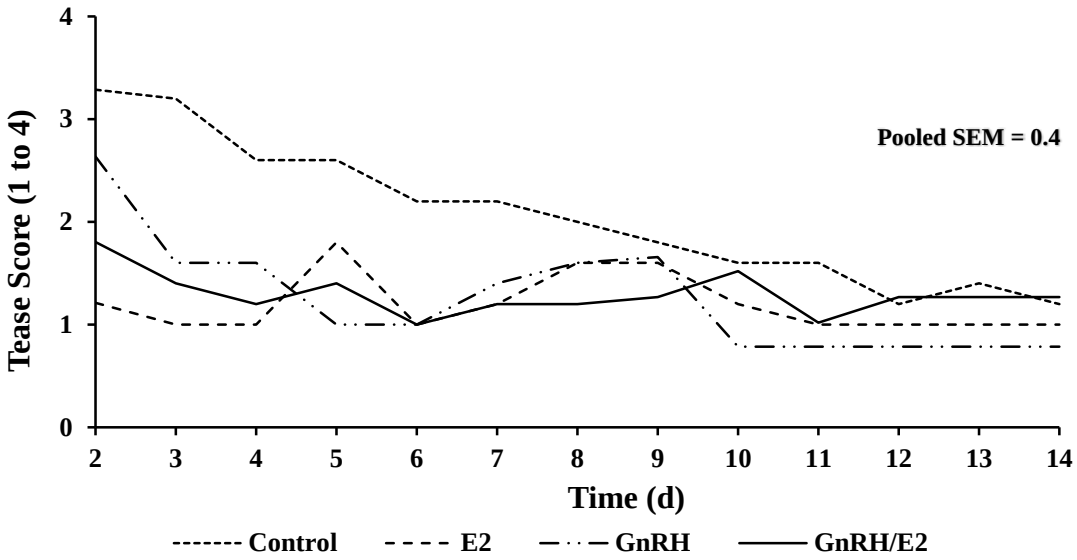

Fig. 5.5. Least squares mean teasing scores beginning on day 2 and continuing through day 14 of the experiment. Mares were teased in small groups at the same time daily in a large pen adjacent to the stallion and separated by solid panels. Treatment x day ($P <$ 0.03)

5.4 Discussion

Results of this study indicated that chronic treatment of winter anovulatory mares with estradiol alone had no effect on circulating concentrations of LH or follicular development. Although 1 mare exhibited a profound and sustained increase in circulating LH relative to other estradiol-treated mares, it is uncertain whether this was a reflection of individual responsiveness to the treatment or coincidental neuroendocrine transition. Because of the rapid increase in LH observed in this mare, it is tempting to speculate that individual mares may respond at different times to this treatment, depending upon endogenous secretion of basal GnRH or the ability of estradiol to trigger such an increase. In any case, such variation may explain some of the contradictions in

earlier reports [10,73] and may also indicate that both dose of estradiol and duration of treatment could contribute to variability observed.

While estradiol alone did not increase circulating concentrations of LH, the current study demonstrated that treatment with estradiol enhanced responsiveness to chronic GnRH treatment designed to increase basal secretion of LH shown to stimulate growth of a preovulatory follicle. The ability of estradiol to increase pituitary responsiveness to GnRH has been shown previously in other species [85,94,95] and in some reports in the mare. In the latter case, chronic estradiol treatment failed to enhance the ability of a GnRH agonist to increase secretion of basal LH. This failure likely occurred in previous studies because of desensitization of GnRH receptors in response to continuous treatment with GnRH agonists which does not occur readily when native GnRH is employed [73]. Nonetheless, enhanced responsiveness to challenge with a bolus injection of GnRH was reported [73]. The effectiveness of continuous treatment with native GnRH alone to increase circulating concentrations of LH in anovulatory mares has been well documented and confirmed herein [53,82,89,96]. Although not examined in the mare, data from numerous other female mammals indicate that estradiol can up-regulate the GnRH receptor [85,97,98]. It is not possible to discern from the present study whether estradiol enhanced responsiveness to GnRH by increasing GnRH receptors or by direct hypothalamic effects or both. However, since estradiol alone did not appear to have significant effects on basal secretion of LH, we interpret our results to indicate that estradiol increased responsiveness to GnRH by effects on pituitary receptors to GnRH or by increasing pituitary content of LH. In an evaluation of

concentration and content of pituitary GnRH receptors, Hart et al. [18] observed no significant differences across reproductive seasons. Similarly, Silvia et al. [43] found no differences in concentration of GnRH receptors from the winter anovulatory period to the breed season. However, there was a decrease in pituitary content of GnRH receptors during the anestrous period relative to the follicular phase of the estrous cycle. In the ewe, an increase in GnRH receptors in cultured pituitary cells has been shown following incubations with estradiol [85]. An increase in releasable LH may also stem from increased synthesis of LH occurring during increasing photoperiod and potential reproductive transition at the neuroendocrine level [11]. The latter infers increased basal secretion of GnRH that is required to stimulate synthesis of gonadotropin.

While concentrations of LH differed among treatment groups, mean follicular size did not differ over the the14-d experimental period (Fig. 5.4.). Although the current work was not designed to provide sustained stimulation past 14 d, increases in LH in response to continuous treatment with native GnRH requires approximately 3 wk in order to effect the development of a preovulatory follicle in 85% of treated mares [53,89]. Ovulations did occur in both the GnRH and the GnRH/E2 group during the trial but, due to individual mare variation, 14 d was not adequate to make significant conclusions regarding ovarian effects of combining estradiol with GnRH treatments. One caveat to this approach is that estradiol has been shown to suppress FSH concentrations in several female mammals [99-101], including the mare [73]. Thus, it is possible for estradiol to suppress follicle growth while at the same time enhancing secretion of LH. This needs to be evaluated in further experiments since recruitment of

antral follicles for final maturation in response to LH for any of these treatments to be effective.

Within a few days following the initiation of treatments, the majority of the mares from the Estradiol, GnRH/E2 and GnRH groups expressed a teasing score of 1. Behavior scores were somewhat variable in each of the three groups with some of the mares maintaining a teasing score of 1 for the entirety of the study, while others expressed estrous behavior more infrequently. In estradiol-treated groups, such a response was to be expected given the high pharmacological dose and stimulatory effects of exogenous estradiol on the CNS [102]. The expression of estrus in mares treated only with GnRH (GnRH group) was likely due to steroidogenic activity in small follicles similar to that seen by Thorson et al. [75] and others following native GnRH treatment [51,52,80]. One report suggested that this may be related to stimulation of angiogenic factors associated with follicular growth [71]. However, by the end of the trial, mean teasing scores for mares in the control group did not differ from that of the other treatment groups. This can partially be explained by two of the mares in the control group that had begun to develop large follicles near the end of the study. For mares with only small follicles, such teasing behavior is much less explainable but is commonly reported [103] . It may occur as a result of biostimulation when exposed to a stallion, the approach of vernal transition and increased steroidogenic activity of small follicles, or other social factors that are not well-characterized.

In conclusion, the current study indicates that estradiol plays a role in modulating pituitary responsiveness to GnRH in the mare. In the current work, this occurred during

increasing photoperiod. However, it is likely that the same response would have occurred at other times in the presence of significant GnRH stimulation. Although not the main focus of the study, ovarian data hinted that estradiol treatment at a pharmacological dose may not provide advantages to the advancement of ovarian recrudescence. Additional work is required in order to clarify this issue. Such investigations could provide a better understanding of the underlying mechanisms mediating the transition from anovulatory to ovulatory status and the development of additional approaches for hastening reproductive transition.

CHAPTER VI

CONCLUSION

The results of studies reported herein have expanded upon RF9's action in the mare relative to previously-published data. Experiments confirmed that RF9 increases secretion of LH in the winter anovulatory mare and that estradiol pretreatment enhances responsiveness to RF9. In addition, we demonstrated that the effect of RF9 on secretion of FSH is similar to its effects on LH. Clearance rate studies revealed that the terminal half-life of RF9 in the circulatory system of the mare is approximately 40 min. Thus, its value as a pharmacological agent may be limited to its ability to cause an acute response unless practical and economical methodologies for prolonging its action are developed. Finally, we demonstrated in vivo that estradiol enhances anterior pituitary responsiveness to GnRH in the winter anovulatory mare. This effect was realized when estradiol was injected daily in conjunction with chronic, subcutaneous infusion of GnRH. The latter treatment approach has been demonstrated previously to rapidly accelerate the timing of the first annual ovulation in 85% of seasonal anovulatory mares [8]. However, other issues related to effects of estradiol on secretion of FSH and ovarian follicular development must be examined in greater depth before a role for estradiol in this setting can be confirmed.

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APPENDIX

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:

   A. Day 1: Begin Assay

      1. NSB – 500 µL of 1% PBS-EW (egg white)
      2. 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

B. 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

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

Equine FSH Radioimmunoassay

1. Iodinated Product: Iodination grade eFSH (AFP-5022B)

2. Antibody: Anti-equine FSH (AFP-2062096). Dilution 1:12,500

3. Standards (stds): Iodination grade eFSH (AFP-5022B; 0.5 – 25 ng/mL)

4. References (ref): eFSH added to equine plasma

5. RIA procedure:

A. 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 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-eFSH (diluted in PBS-EDTA + 1:400 NRS) into all tubes except NSB and TC tubes.
8. Vortex tubes briefly and incubate for 1 h at room temperature.
9. Pipette 100 µl 125I-eFSH (20,000cpm/100 µl diluted in 0.1% PBS-EW) to all tubes.
10. Vortex tubes briefly and incubate for 24 h at 4°C.

B. Day 2: Add Second Antibody

1. Keep all test tubes and reagents on ice during all procedures.

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

C. Day 4: Take Off Assay

1. Keep all test tubes and reagents on ice during all procedures.
2. Add 3.0 mL ice cold PBS (0.01 M; pH 7.0) to all tubes except TC tubes.
3. Centrifuge tubes for 1 h at 4°C at 3600 rpm.
4. Decant supernatant.
5. Count radioactivity of each tube using a gamma counter.

Extraction and LC/MS protocol for curve

Tube	#1	#2	#3	#4	#5	#6	#7	#8
[RF9]	2ng/mL	5ng/mL	10ng/mL	20ng/mL	50ng/mL	100ng/mL	300ng/mL	500ng/mL
PBS	98ul	95ul	90ul	80ul	50ul	0ul	85ul	75ul
D1	2ul	5ul	10ul	20ul	50ul	100ul	----------	----------
WS	----------	----------	----------	----------	----------	----------	15ul	25ul
Plasma	100ul	100ul	100ul	100ul	100ul	100ul	100ul	100ul

Reagents

A. Stock = 2ug/mL RF9 in PBS

B. Working Stock = 100ng/mL RF9

C. Extraction solvent = Acetonitrile w/ 0.1% Formic Acid

D. Resuspension Solvent = mEQ water w/ 10% acetonitrile and 0.1% Formic Acid @100ng/mL testosterone-D3

Procedures

1. Pipette PBS to labeled Eppendorf tubes (see above table)
2. Pipette RF9 (see above table)
3. Pipette 100 µL of plasma
4. Vortex briefly, allow few minutes to equilibrate
5. Pipette 500 µL, acetonitrile-0.1% formic acid (standard and unknowns treated the same from this point on)
6. Vortex for 30 sec. and let set for 2 min to equilibrate
7. Centrifuge 15min @ 13K rpm
8. Extract 400ul supernatant, take care not to disturbing pellet
9. Spin Vac w/ heat for 1h 20 min.
10. Pipette 100ul mEQ water-10%acetonitriloe-0.1% formic acid + testosterone-D3
11. Vortex a minimum of 30 sec.
12. Sonicate in water bath for 10 min.
13. Centrifuge 5 min. @13K rpm
14. Extract 40ul for single injection or 80ul for duplicates and pipette into disposable glass nipple tube, insert nipple tube into reusable glass vial and screw penetrable cap down tight