THE EFFECTS OF ESTRADIOL-17BETA IN MODULATING PITUITARY-OVARIAN RESPONSIVENESS TO CONTINUOUS INFUSION OF GONADOTROPIN-RELEASING HORMONE IN WINTER ANOVULATORY MARES

A Thesis

by

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

MASTER OF SCIENCE

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December 2017

Major Subject: Physiology of Reproduction

ABSTRACT

Continuous infusion with native gonadotropin-releasing hormone (GnRH) restores follicular growth and ovulation in approximately 85% of winter anovulatory mares. However, the potential role of estradiol-17β (E2) in enhancing pituitary responsiveness to GnRH during this transitional process is unclear. Moreover, the contribution of follicle-stimulating hormone (FSH) to the overall response has not been studied. Objectives herein were to test the hypotheses that (1) continuous treatment with either a low or high dose of E2 enhances pituitary responsiveness to continuous infusion of native GnRH, and (2) the pattern of FSH secretion mimics that of LH. Twenty-four winter anovulatory mares were stratified by body weight and body condition score, and assigned randomly to 1 of 4 treatments (n = 6/treatment) in early February: 1) Control; Silastic® subcutaneous (S.C.) sham pump, 2) GnRH; native GnRH in saline via 14-day Alzet® osmotic pump (100 μg/h, S.C.), 3) GnRH + High E2; GnRH as in 2 plus Compudose® E2 implant, (25.7-mg, S.C.), and 4) GnRH + Low E2; GnRH as in 2 and 3 plus 1/4-length E2 implant (6.425-mg, S.C.). Plasma concentrations of LH, FSH, E2, estrone sulfate, and progesterone were measured in daily blood samples. Mares developing 35-mm follicles were induced to ovulate with human chorionic gonadotropin (hCG). Mares not ovulating within 14 days received a second 14-day GnRH pump. Although acute increases in uterine edema observed within 48 hours indicated implant functionality, plasma E2 and estrone sulfate were increased primarily in association

with a large, dominant follicle. Plasma LH was increased in all GnRH-treated mares, regardless of ovarian response, but was unaffected by E2. By Day 28, 6/6 GnRH, 5/6 GnRH + Low E2, 2/6 GnRH + High E2, and 0/6 Control mares developed a 35-mm follicle and were induced to ovulate. A peak of FSH was observed in response to all GnRH treatments at 24 hours, with plasma FSH returning to baseline by day 4. Therefore, the hypotheses were not supported as the subcutaneous E2 implants failed to enhance responsiveness of mares to GnRH and the release of FSH in response to GnRH infusion did not mimic the LH release pattern.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Gary L. Williams, and my committee members, Dr. Rodolfo C. Cardoso, Dr. Thomas H. Welsh Jr., and Dr. Katrin Hinrichs, for their continued guidance and support throughout the course of this research.

A thanks also goes to all of my friends and fellow graduate students whom helped make the master's journey an enjoyable one. I would not have been able to make it through with a smile on my face without you all.

Finally, an overwhelming thank you to my family for always supporting me, no matter what path I chose to take. Livestock reproduction may be a far cry away from your comfort zone, but you all have embraced my passion for the industry with open arms. I cannot thank you enough.

CONTRIBUTORS AND FUNDING SOURCES

This work was supported and supervised by a thesis committee consisting of Dr. Gary L. Williams, Dr. Rodolfo C. Cardoso, and Dr. Thomas H. Welsh, Jr. of the Department of Animal Science and Dr. Katrin Hinrichs of the Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine & Biomedical Sciences.

Plasma estrone sulfate concentrations were determined by RIA performed by Dr. Scott Jaques at the Texas A&M University Veterinary Medical Diagnostic Laboratory in College Station, TX.

All other work conducted for the thesis was completed by the student, independently.

The research documented herein was supported by the Link Equine Research Fund, Texas A&M University.

NOMENCLATURE

GnRH	Gonadotropin-releasing hormone
E2	Estradiol-17β
EB	Estradiol benzoate
LH	Luteinizing hormone
FSH	Follicle-stimulating hormone
P4	Progesterone
CL	Corpus luteum
CH	Corpus hemorragicum
OVX	Ovariectomized
BW	Body weight
BCS	Body condition score
RIA	Radioimmunoassay
IGF-1	Insulin-like growth factor-1

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I. INTRODUCTION

The horse is a seasonally reproductive livestock species in which estrous cyclicity coincides with long-day photoperiod and is observed during the late spring, summer and early fall months. Transition from the anovulatory to ovulatory state begins, in general, around the time of the vernal equinox (March 22 and September 22 in the northern and southern hemispheres, respectively). On average, mares in North America exhibit their first ovulation in early April (April 7 ± 9.1 days) [1,2]. By the summer solstice (June 20-21), 95-100 % of mares exhibit regular 19- to 24-day estrous cycles until approximately the time of the autumnal equinox (September 22), at which time the proportion of mares ovulating and peripheral luteinizing hormone (LH) concentrations decline slowly. Individual mares in certain years will continue to ovulate well into the fall and early winter, with 15-30% of mares in a given year continuing to cycle throughout the winter period [3,4].

Many horses are valued for their performance and recreational purposes, placing emphasis on the pedigree of the animal. Pedigree value is increased by accredited registration of the horse and its progeny. In the northern hemisphere, the majority of breed registries employ a universal birthdate of January 1. Thus, horses are officially considered a year older on each subsequent January 1, regardless of actual birth date. The universal birthdate promotes an incentive for owners and breeders to create an operational breeding season that is in advance of the natural season, and its transitional period, by approximately 2 months [4]. Foals born earlier in the year have an advantage

*Parts of this chapter have been reprinted from "Secretions of gonadotropins is differentially regulated by continuous infusion of native GnRH and is unaffected by exogenous estradiol in winter anovulatory mares" by O'Neil MM, Scarpa JO, Cardoso RC, Williams GL. J Eq Vet Sci 2017; 52: 112. Copyright Elsevier 2017.

in size and maturity compared to those born later in the year. A variety of managerial and pharmacological treatments have previously been investigated for their capability to artificially hasten the onset of cyclicity and ovulation in winter anovulatory mares, and have been evaluated for their practicality in a normal management setting as described below.

II. REVIEW OF LITERATURE

2.1. Gonadotropin profile of the mare

During the luteal phase and early follicular phases of the estrous cycle, follicle-stimulating hormone (FSH) is secreted to stimulate a cohort of follicles to grow. Follicles are selected and one, sometimes two, follicles undergo deviation, whereby the follicle(s) grow in diameter and develop endocrine capacities necessary for ovulation [2,4]. As follicle selection and deviation take place, the dominant follicle (or follicles where 2 large follicles are selected) gains the capacity to synthesize and secrete inhibin from granulosa cells. This ovarian glycoprotein acts at the pituitary to inhibit secretion of FSH and accounts for the decline of circulating FSH as the dominant follicle matures. Other intra-follicular factors, including insulin-like growth factor 1 (IGF-1) and follistatin, also contribute to negative feedback regulation of FSH [5]. The dominant follicle gradually synthesizes and secretes increasing quantities of estradiol-17β (E2), peaking approximately 4 to 6 days before ovulation [6]. Unaffected by inhibin and under positive influence from GnRH, LH pulses become more frequent after luteal regression and, together with increasing amplitude, increases circulating concentrations of LH to values that result in ovulation of the preovulatory follicle [7]. After ovulation occurs, LH triggers the luteinization of the follicle, initially forming a corpus hemorrhagicum and then a corpus luteum (CL). The CL functions to synthesize and secrete progesterone (P4), resulting in circulating concentrations indicative of a functional CL (> 1.0 ng/mL) [8]. At CL regression, progesterone concentrations decline to a baseline well below 1.0 ng/mL where it remains until formation of the next CL [8].

2.2. Neuroendocrine seasonality

During the winter months, or short days, the nightly duration of melatonin secretion from the pineal gland is increased relative to that occurring during long-day photoperiod. In the mare, the pineal gland entrains the photoperiodic circadian rhythm as demonstrated by the lack of response to photoperiod after pinealectomy or superior cervical ganglionectomy [9,10]. The seasonal disruption in reproductive cyclicity also depends on a photorefractory response, photoperiodic response history, and an established photosensitive period [11]. During periods of decreased photoperiod, LH β-subunit synthesis is down-regulated and LH secretion is suppressed [12]. Interestingly, FSH in equids does not exhibit a seasonal decline in the peripheral circulation [12]. Due to the suppression of LH synthesis and secretion, approximately 85% of light horse mares enter a period of anovulation in the winter, and ovarian follicles do not grow to ovulatory size [4]. During the spring transitional period, as defined by the period of lengthening photoperiod, the proportion of mares ovulating directly corresponds to the increase in peripheral concentrations of LH, and ovarian activity resumes as a result of the effects of LH on follicle maturation and ovulation [13]. At this time, the pattern of ovarian follicular growth undergoes a drastic change as follicular waves emerge in which a large follicle is selected, deviates from the rest of the follicles in the wave, and develops into a dominant follicle capable of undergoing ovulation. During the process of selection and deviation, the two larger follicles generally develop in tandem, increasing in diameter until one follicle is selected and undergoes deviation at approximately 22

mm. The subordinate follicle regresses and becomes atretic, while the selected follicle continues to mature and reaches periovulatory status at approximately 30 mm [4].

2.3. Artificial photoperiod

Photoperiod has been well documented as the primary factor regulating seasonal reproduction in the horse [2,4,9,10,14,15]. Manipulation of light exposure has varying effects on hastening the onset of ovulation in the winter anovulatory mare. In an early study, artificially exposing mares to a total of 16 hours of light and 8 hours of dark in a 24-hour period was shown to be effective in accelerating the onset of first ovulation in the mare when begun December 1, although the exact ratio of light to dark could vary 1 to 2 hours [14]. A protocol termed flash lighting has also been effective in stimulating the advancement of seasonality and requires less electricity and light exposure. Beginning around 4:00 am, or approximately 9 hours after dusk, the protocol requires mares to be subjected to a 1-hour pulse of light, in addition to the natural exposure to daylight [14].

More recently, a technology called Equilume has been developed (Equilume Ltd, Co. Kildare, Ireland). A mask, similar to those used in racing, is equipped with a quarter-cup over the right eye. The cup houses a blue LED light that is programmed to activate long enough to expose the eye to the full 16:8 hour light to dark ratio and has been shown to stimulate early transition and cyclicity comparable to the traditional artificial lighting protocol [16]. Though this technology eliminates the necessity of costly infrastructure for broodmares, the masks are currently battery operated and require a

significant investment of at least $400 USD per unit, which may limit industry-wide application.

Regardless of the lighting protocol, artificial lighting regimens have been shown to produce variable responses in studies involving large numbers of mares and require significant investments in labor, time, and infrastructure to implement [17]. Thus, other options such as use of pharmacological agents have been pursued.

2.4. Pharmacological therapies to advance seasonality

2.4.1. Melatonin

As described previously, melatonin is believed to be the primary photoperiodic-driven hormonal cue that modulates GnRH and gonadotropin secretion in response to photoperiod. In ewes, melatonin microimplants inserted into the pre-mammillary hypothalamic area were able to stimulate the secretion of LH, and have the ability to hasten onset of seasonal estrous activity [18]. However, when ovariectomized (OVX) pony mares were treated with subcutaneous melatonin implants during the breeding season, no effects on circulating concentrations of LH, E2, or P4 were observed [19]. In intact mares, secretion of GnRH and LH was increased and the last ovulation of the breeding season was delayed following treatment with subcutaneous melatonin implants [19]. However, in the same study, long-term treatment with melatonin delayed significantly the onset of the subsequent breeding season, suggesting that exogenous melatonin treatment can alter the circannual rhythm [20]. Other studies involving treatment with melatonin have resulted in variable effects on secretion of LH and

evidence for a potential interaction with prolactin, suggesting that melatonin treatment alone does not accelerate consistently the timing of reproductive transition and first ovulation in the winter anovulatory mare [21,22].

2.4.2. Opioids

The secretion of GnRH, and thus gonadotropins, is reduced in the presence of heightened opioid tone within the hypothalamus [23,24]. Evidence suggests that endogenous opioid peptides may play a role in regulating equine seasonal reproduction. After administration of the opioid antagonist, naloxone, circulating concentrations of LH increased significantly in winter anovulatory mares [25-27] and this response may be related to dose [26] and season [28,29]. A bolus, intravenous (iv) injection of naloxone during the breeding season had a similar effect, but increased secretion of GnRH and FSH was also documented [30]. Davidson et al. suggested that due to the inhibitory nature of endogenous opioids on gonadotropin secretion, reduced opioid tone could explain the minority of mares that continue to cycle throughout the year [29]. Although data suggests endogenous opioids play a role in modulating GnRH and gonadotropins, this role and how it relates to reproductive seasonality in the mare remains to be completely defined.

2.4.3. Catecholamines

After it was discovered in 1990 that concentrations of catecholamines, including dopamine, were greater in cerebrospinal fluid during the anovulatory season in mares compared to the breeding season [31], dopamine antagonists were tested for their ability to advance vernal reproductive transition. While some studies have shown that dopamine

antagonists can stimulate follicular development and decrease the time to first ovulation [32-34], others including those involving long-term application, have reported limited to no effect on gonadotropin secretion or ovulation [33-37]. A recent study, however, reported that pre-treatment with estradiol benzoate (EB) prior to injection of sulpiride, a dopamine antagonist, hastened the onset of first ovulation and increased both LH and prolactin concentrations in seasonally anovulatory mares compared to mares given sulpiride alone [38]. In that same study, it was hypothesized that the pre-treatment of EB increased the response of winter anovulatory mares to the dopamine antagonist [38].

Despite inconclusive evidence for the ability of dopamine antagonists to advance the onset of ovulation, concentrations of prolactin, whose secretion is negatively controlled primarily by dopamine, increase with the lengthening photoperiod and heightened temperatures associated with onset of the breeding season [33,37,39]. Prolactin receptor immunohistochemical cell staining has been reported to be most intense in large antral follicles, providing further evidence of a possible mechanistic role of prolactin during follicular maturation and potentially ovulation [40]. It has been suggested that prolactin is needed for LH receptor numbers to be adequate for ovulation in the female rat [41], though this potential role of prolactin has not been described in the mare.

Exogenous prolactin treatment in winter anestrous mares induced early vernal hair shedding, estrus, and ovulation; however, with little effect on concentrations of LH compared to untreated, control mares [42], supporting the possibility that prolactin works via receptor population. A role of prolactin in seasonal reproductive cyclicity is

supported by observations that lactotroph populations in the pars distalis are more numerous during the breeding season compared to the non-breeding season in the mare [43]. There is also evidence that gonadotrophs in the pars distalis colocalize with lactotrophs, indicating the possibility of cross-talk between the two cell populations [43,44]. In the ewe, increased lactotroph cytoplasmic area, greater rough endoplasmic reticulum populations, and increased density of prolactin-secreting granules are also reported during the breeding season when compared to the non-breeding season [45]. Thus, it has been proposed that increased circulating concentrations of prolactin, accompanied by parallel increases in dopaminergic cell populations, may account for the mare's ability to reduce pituitary desensitization of the GnRH receptor during the breeding season [46].

The ability of GnRH to stimulate prolactin secretion has been investigated in ovine pituitary cell cultures. In those experiments, GnRH stimulated a dose-dependent increase in prolactin release during the breeding season in the ewe, a short-day reproductive species [47]. This effect was completely inhibited by a dopamine agonist [47]. The relative impact of these interrelationships in the mare, however, has yet to be investigated.

2.4.4. GnRH

It has been well established that an endogenous, intermittent hypophysiotropic signal of GnRH is required to optimally stimulate the synthesis and secretion of LH necessary for ovulation [48,49]. Native GnRH and several synthetic GnRH agonists (buserelin, deslorelin, histerelin, goserelin) have been used both experimentally and

clinically to induce ovulation in cattle (native GnRH) and horses (GnRH agonists). In winter anovulatory and transitional mares, it was hypothesized that these analogues could stimulate the hypothalamic-pituitary-ovarian axis sufficiently to elevate peripheral concentrations of LH to induce follicular development and ovulation. Although buserelin injections have been effective in accelerating the time of ovulation and pregnancy in the winter anovulatory mare compared to untreated mares [50], later studies revealed the agonists' tendency to temporarily desensitize pituitary gonadotropes when given in either repeated injections or implant [51-53]. This most likely occurs due to the much greater affinity of the agonists for the gonadotrope receptor resulting in delayed clearance and related receptor down-regulation [51-53]. Temporary pituitary desensitization was further supported when a single implant of deslorelin was efficient in stimulating ovulation, but prevented LH and FSH response to a GnRH challenge, with suppression lasting until 10 days post-ovulation [54]. Despite the apparent ability of GnRH agonists to stimulate ovulation in winter anovulatory mares, the pituitary desensitization and subsequent suppression of gonadotropins makes synthetic agonists undesirable for consistently hastening the onset of regular estrous cyclicity in the mare.

Native GnRH does display the same capacity to down-regulate receptors compared to GnRH agonists in the winter anovulatory mare. Early studies showed that hourly, intravenous injections of native GnRH in doses ranging from 2 to 20 μg/hour were able to significantly increase peripheral concentrations of LH and accelerate ovulation in seasonally anestrous mares [55,56]. Though this method of delivery best mimics the endogenous hypophysiotropic signal [48,49,55,56], hourly pulsatile

injections are not practical. Therefore, numerous studies have investigated the efficacy of subcutaneous delivery of the native hormone. Although continuous subcutaneous infusion of native GnRH does not increase peripheral LH to breeding season values, it is capable of elevating concentrations of LH in both the winter anovulatory and cycling mares [57,58]. When administered at a rate of 100 μg/hour using an osmotic pump, native GnRH hastened the onset of first induced ovulation and pregnancy by approximately 2 months (response seen in 85% and 79% of winter anovulatory mares, respectively), compared to controls [59,60]. It is unclear, however, why 10-15% of winter anestrous mares failed to respond to the constant GnRH stimulus, requiring further investigation.

The method of delivery using the osmotic pump consists of surgically implanting it subcutaneously in the neck of the mare, cranial to the scapula [58-60]. Until a more user-friendly delivery method is devised that can be practically implemented in the equine breeding industry, its adoption will continue to be hindered.

2.4.5. Estradiol

Estradiol-17β is the primary biologically-active estrogen synthesized in mammalian systems and is produced in relatively large quantities by “estrogen-active”, dominant follicles in the mare that are approximately 20 mm or greater [6]. Circulating concentrations of E2 increase significantly about 6 to 8 days prior to ovulation, peaking approximately 2 days before ovulation [4]. The peak in E2 precedes the ovulatory peak of LH, suggesting a positive feedback of E2 on the hypothalamic-pituitary-ovarian axis during estrus [61], similar to that characterized in other mammalian females [62].

Although the proposed manner of action remains to be defined in the mare, several studies have provided evidence that E2 stimulates increased pituitary content and peripheral concentrations of LH [63], but may create this effect by a method other than enhancing the pituitary response to GnRH [64]. Pre-treatment of mares with exogenous E2 prior to GnRH treatment enhanced circulating LH concentrations compared to those for controls [65]. However, in a more recent study, increasing doses of E2 led to decreased concentrations of LH in the estrual mare [66]. Studies in ovariectomized (OVX) mares have also resulted in inconclusive or ambiguous results. Miller et al. [67] reported that E2 decreased both LH and FSH initially, followed by a brief increase 12 hours later. In another study involving OVX mares during the vernal transitional period, E2 had no effect on the secretion pattern of FSH, but had a positive effect on concentrations of LH [63]. When E2 was given twice daily in the OVX early transitional mare, there was a significant increase in both α and β LH subunit synthesis within the pituitary [68].

The effects of coincident treatment with E2 and GnRH have also been examined [65,69,70]. However, the interpretation of results is somewhat complicated because GnRH agonists were employed in several of the studies. As a result, it is difficult to fully ascertain the independent effects of E2 because of the possibility of GnRH receptor down-regulation in mares treated chronically with these agonists. A recent preliminary study performed in our laboratory tested the effects of continuous infusion of native GnRH simultaneously with daily injections of exogenous E2 [71]. Mean circulating concentrations of LH were increased slightly but significantly after 7 days in E2-treated

mares compared to GnRH only. Results were interpreted to indicate the possibility of enhanced sensitivity of gonadotropes to GnRH, in contrast to previously findings [63].

To the best of our knowledge, apart from the preliminary study described above, secretion of LH in response to physiologic versus pharmacologic doses of continuous exogenous E2 administered coincident with the continuous, subcutaneous infusion of native GnRH has not been investigated. In addition, the secretion patterns of FSH in response to chronic treatment with native GnRH, and the interactive effects of E2, have not been characterized in the winter anovulatory mare. A greater understanding of FSH secretory dynamics in response to continuous GnRH treatment may yield a better understanding of the physiology and endocrinology of the 10-15% of the winter anovulatory mare population that fails to respond to this treatment. Based on the foregoing knowledge, we designed a project to investigate the effectiveness of continuous, subcutaneous E2 in enhancing pituitary responsiveness of GnRH and to observe the resulting secretion pattern of FSH as it compares to LH in response to native GnRH or native GnRH + E2.

III. DIFFERENTIAL REGULATION OF GONADOTROPINS IN RESPONSE TO CONTINUOUS INFUSION OF NATIVE GnRH IN THE WINTER ANOVULATORY MARE: EFFECTS OF CONTINUOUS TREATMENT WITH LOW- OR HIGH-DOSE ESTRADIOL-17β IMPLANTS*

3.1 Introduction

Mares are long-day breeders, with resumption of pituitary-ovarian activity associated with increasing photoperiod following the spring equinox. Due to a widely-employed universal birthdate of January 1 in the northern hemisphere (July 1 in the southern hemisphere) by numerous breed registries, there is managerial incentive for professional breeders to accelerate the natural transition period by approximately 2 months [4]. Foals born as close to, but after January 1, are more mature early in their second and third years of life. Thus, they have a competitive advantage over those born later in the season and are usually more valuable at auction [4,72]. Moreover, mares that foal earlier in the season have the opportunity to be bred back at an earlier date, thus enhancing the potential for production of another foal early in the subsequent season [2].

During the winter months, 85% of standard horse mares enter a period of winter anovulation [4] due to marked declines in pituitary synthesis and secretion of LH [12,73]. Overall mean circulating concentrations of FSH are unaffected by changing season [12,74,75]. However, increments in serum/plasma FSH are clearly required for the recruitment of each new follicle wave during established cycles, peaking at about the

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time of dominant follicle deviation [2,4]. During the winter months, anovulatory follicular waves can occur, but follicular diameters do not typically exceed 20 mm, and follicles become atretic [75]. It is not until the vernal transitional period that selected dominant follicles gain the capacity to secrete E2, reach preovulatory status, and exert positive feedback to induce the ovulatory surge of LH [75, 76].

The exogenous administration of GnRH agonists, such as buserelin and deslorelin, has been used successfully in mares to stimulate secretion of LH and induce ovulation of large follicles in winter anestrus [50]. However, when administered chronically as an injection or implant, GnRH agonists often lead to temporary pituitary desensitization of the gonadotropes, making them a less than optimal method of advancing reproductive transition despite variable success [50-53]. Notably, chronic administration of native GnRH in the mare is less likely to result in marked desensitization, particularly if administered during the non-breeding season when endogenous secretion of GnRH is low [55-60]. Hourly, intravenous injections of as little as 2 μg have been shown to markedly increase concentrations of LH and stimulate follicular development, maturation, and ovulation in winter anovulatory mares. However, hourly injections have limited practicality for professional breeders [55-56]. Although continuous infusion of native GnRH via an osmotic pump does not optimize pituitary responses or result in breeding season concentrations of peripheral LH, it is an effective alternative for increasing circulating concentrations of LH to values that rapidly stimulate development of a large follicle. This facilitates natural breeding or AI coupled with induced or natural ovulation up to 2 months earlier than typically expected [57-60].

However, approximately 15% of mares consistently fail to respond to this treatment [57-60]. Therefore, further studies are required to determine the basis of its failure. In this context, the pattern of FSH secretion and its role in modulating ovarian responsiveness to continuous infusion of native GnRH has not been studied. Moreover, there is conflicting evidence regarding the ability of E2 to increase secretion of LH during vernal transition or in response to continuous GnRH treatment [56,65]. In a preliminary study in our laboratory, twice daily intramuscular injections of EB, coincident with continuous infusion of native GnRH, led to modestly increased peripheral concentrations of LH compared to GnRH alone after 7 days [71].

The objectives of the experiment reported here were to test the hypotheses that 1) continuous treatment with either a low (peripheral target ~ 20-25 pg/mL) or high (peripheral target of ~100 pg/mL) dose of E2 will enhance pituitary responsiveness to continuous infusion of native GnRH in winter anovulatory mares, and 2) the FSH response to GnRH or GnRH plus E2 treatments will mimic that of LH until deviation is reached by a newly-recruited large follicle.

3.2 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 (AUP #2016-002A).

3.2.1 Animals

Twenty-four non-pregnant light horse mares, the majority being American Quarter Horses, were maintained on mixed grass pasture and supplemented with Coastal Bermuda or Alfalfa grass hay in order to maintain a body condition score (BCS) of a 5 to 6 (on a 1 to 9 scale) [77] at the Texas A&M Agrilife Research Station in Beeville, Texas.

3.2.2 Preliminary experiment

In December, prior to the start of the formal study, a preliminary experiment was performed to confirm estrogenic effects of both a full and 1/4 E2 implant (Compudose® implants; Elanco Animal Health, Greenfield, IN) placed subcutaneously in the neck of winter anovulatory mares under natural photoperiod. Four non-pregnant mares, confirmed to be anovulatory, were assigned randomly to 1 of 2 treatments (2 mares/treatment): 1) a full estradiol-17β implant (Compudose®; 25.7-mg, s.c.) or 2) a precisely cut 1/4 E2 implant (Compudose®; 6.425-mg, s.c.). Two untreated mares served as controls and were monitored for direct comparison. Implants were surgically inserted subcutaneously and left in place for 4 days. Surgical procedures for placement and removal were performed as described below. Following implantation, mares treated with both implant sizes developed extensive uterine edema within 48 hours that was maintained until implants were removed, as seen on transrectal ultrasonography, indicative of elevated circulating E2 [78], whereas no edema was observed in controls.

3.2.3 Experimental procedures

In January, mares were selected for use in the study based on confirmation of winter anovulatory status as determined by transrectal ultrasonography performed every 2 to 3 days for 3 weeks before the start of the experiment. Criteria for confirming the anovulatory state included the absence of a CL and no follicle exceeding 29 mm in diameter for the 3-week period. A retrospective analysis of plasma concentrations of progesterone was also performed in blood samples collected immediately following ultrasonography. Body weight (BW) of all mares was estimated using a BW tape based on heart girth circumference and BCS was assigned on a 1-9 scale by a single individual.

During the first week of February, 24 mares confirmed to be anovulatory were stratified by age, BCS, and BW and assigned randomly to 1 of 4 treatments (n = 6/treatment): 1) Control; Silastic® s.c. sham pump, 2) GnRH; native GnRH in saline via 14-day Alzet® osmotic pump (100 μg/hour, s.c.), 3) GnRH + High E2; GnRH as in 2 plus Compudose® estradiol-17β implant, (E2 implant; 25.7-mg, s.c.), and 4) GnRH + Low E2; GnRH as in 2 and 3 plus 1/4-length E2 implant (6.425-mg, s.c.). Jugular blood samples were collected daily for assay of gonadotropins, E2, estrone sulfate, and P4. Follicular development and uterine edema were monitored every other day until the development of a 30-mm follicle was detected via transrectal ultrasonography. Once a 30-mm follicle was detected, mares were considered periovulatory and monitored via transrectal ultrasonography every day until the development of a 35-mm follicle. Upon detection of a 35-mm follicle, mares were treated with human chorionic gonadotropin (hCG; Chorulon; MSD Animal Health, Wellington, New Zealand; 2000 IU, I.M.) to induce ovulation. Each Alzet pump (model 2ML2; DURECT Corp., Cupertino, CA) was

filled with a solution containing GnRH (20 mg/mL; ProsSpec- Tany TechnoGene Ltd., Rehovot, Israel) in sterile 0.9% saline. Pumps had the potential to function for 14 days at a pumping rate of 100 μg/hour. All sham pumps consisted of Silastic tubing (Dow Corning Corporation, Midland, MI).

3.2.4 Subcutaneous placement of pumps and implants

The experiment was initiated in all mares over 2 consecutive days, with Day 0 on either February 8 or 9. On Day 0, all mares were placed in a stock and sedated with detomidine hydrochloride (Dormosedan; Zoetis U.S., Parsippany, NJ; 20-40 μg/kg BW) if necessary. The area at the base of the neck, cranial to the scapula, was clipped and prepared for aseptic surgery using an iodophor, followed by povidone iodine solution and 70% isopropanol. A local anesthetic (3-5 mL lidocaine hydrochloride) was used and a 2-cm skin incision was made at the base of the neck using a sterile no. 10 scalpel. Using a blunt surgical instrument, a pocket was formed in which the respective treatments were inserted. Mares receiving both E2 and GnRH treatments had 2 separate incision sites with at least a 2-cm distance between the two implants. Each incision was sutured with #2 synthetic sutures that remained in place for 7 days. Mares failing to develop a large follicle and/or to ovulate within the first 14 days had the spent pump removed and a second 14-day pump installed on the opposite side of the neck as described above.

3.2.5 Uterine edema

During each ultrasonographic screening, the uterus was monitored for uterine edema as a physiological indicator of onset of estrogen stimulation [78]. A scoring

system described previously was employed (0 = no edema, 1 = minimal edema, 2 = marked edema, 3 = maximum normal edema, 4 = excessive edema) [78].

3.2.6 Estrous behavior

Estrous behavior was assessed daily by placing 5 to 6 mares in a pen containing a solid-paneled wall adjacent to an intact stallion. Mares were assigned subjective teasing scores ranging from 1-4: 1 = physical rejection denoted by kicking or pinning of the ears, 2 = slight interest or preference, 3 = interest denoted by vulvar winking, 4 = complete breakdown denoted by squatting and/or urination in the presence of the stallion.

3.2.7 Blood sampling

Blood samples (10 mL) were collected daily via jugular venipuncture from each mare beginning immediately following insertion of osmotic/sham pumps and implants. Evacuated tubes (Monoject^{tm}, Covidien, Mansfield, MA) containing disodium ethylenediaminetetraacetic acid (EDTA) were used for blood collection. Samples were placed on ice until transported to the laboratory for centrifugation (562 x g) at 4°C for 20 minutes. Plasma was harvested and stored at -20°C until analysis of hormones by RIA.

3.2.8 GnRH challenge

Mares that failed to develop a 35-mm follicle and thus were not treated with hCG to induce ovulation within the 28-day treatment period (4/6 GnRH + High E2; 5/6 Controls) were evaluated further by administering a GnRH challenge to assess releasable pools of LH and FSH. One day before the challenge, mares were fitted with an intravenous jugular catheter (Model J0458B, Jorgensen Laboratories Inc., Loveland,

CO) for intensive sampling. Jugular catheters were held in place by #1 non-absorptive synthetic suture. At the time of catheter insertion, mares received 30,000 IU of heparin (10,000 IU/mL) in 3mL saline to minimize clotting potential. Mares were maintained individually in holding pens until the GnRH challenge the next day. Immediately before onset of treatment and sampling, 20,000 IU of heparin was administered through the catheter. Gonadotropin-releasing hormone (GnRH; 1 mg) was administered via the jugular catheter in 2 mL of 0.9% sterile, physiological saline (GnRH = 0.5 mg/mL). Prior to collecting each sample, blood was discarded to avoid heparin contamination. Blood samples were collected in 5% EDTA with heparin at -30, 0, 15, 30, 45, 60, 120, and 240 minutes in relation to GnRH administration, and placed immediately on ice. Catheters were flushed with 4-5 mL of heparinized saline (1000 IU/mL) after each blood sample was collected. After the final sample was taken, all jugular catheters were removed and insertion sites cleaned with 70% isopropanol. Blood samples were immediately placed on ice for transport to the laboratory and centrifuged (562 x g) at 4°C for 20 minutes. Plasma was stored at -20°C until hormone analysis.

3.2.9 Hormone analyses

Plasma concentrations of progesterone were assayed in triplicate utilizing a coated-tube (CT) RIA kit (MP Biomedicals, Santa Ana, CA, products #07-270102 and 07-270105) validated in this laboratory. Standards provided by the kit were used (0.15, 0.5, 1.0, 5.0, 20.0 ng/mL) with the addition of two standards (2.0 and 10.0 ng/mL) created by serial dilution of the 80-ng/mL kit standard using the 0.0 ng/mL standard as the diluent. Prior to assay of unknowns, 3 simultaneous validation curves were

performed at 3 incubation times and temperatures to determine optimal incubation condition for equine plasma: 1) 37°C water bath for 2 hours as per kit protocol, 2) 4°C for 16-20 hours, and 3) room temperature (20° C) for 3 hours. Incubation at 4° C for 16-20 hours produced a curve with the lowest variance ratio (2.42) and adequate binding (48%) compared to the 37° C water bath (variance ratio = 28.6, 61% binding) and room temperature (variance ratio = 62.1, 40% binding) incubations, and was therefore utilized for assay of unknowns. Samples were assayed in triplicate at a volume of 100 µL and pipetted into the coated tubes at room temperature. This was followed by addition of radiolabeled tracer (^{125}I-progesterone) at a volume of 1 mL/tube (approximately 40,000 cpm/tube). Tubes were vortexed briefly using a multitube vortexer and incubated for 16-20 hours at 4°C. Tubes were decanted and the bound fraction was counted in a gamma counter for 1 minute per tube. Recovery of added mass (accuracy) was determined by adding known amounts (5.0, and 10.0 ng/mL) of progesterone to a pool of plasma obtained from winter anovulatory mares giving an average observed 88.3% recovery. Due to consistent underestimation of added mass indicated by the percent recovery of added mass, all final values were adjusted accordingly for analysis. The sensitivity was 0.15 ng/mL and the intra- and interassay coefficients of variation were 4.7% and 4.3%, respectively.

Plasma concentrations of LH were assayed in duplicate using a double antibody RIA validated previously in this laboratory [71,79]. Iodinated tracer, standards and references utilized highly-purified equine LH (LH AFP-5130A). Anti-equine LH antiserum (AFP-240580) was used at a dilution of 1:120,000. The sensitivity was 0.25

ng/mL and the intra- and interassay coefficients of variation were 7.1% and 6.0%, respectively.

Plasma concentrations of FSH were assayed in triplicate with a validated double antibody RIA [80] using highly-purified equine FSH (eFSH AFP-8830D) for tracer and standards alongside anti-eFSH antiserum (AFP-2062096) at a working dilution of 1:12,500. The sensitivity was 1.0 ng/mL and the intra- and interassay variation was 6.1% and 6.5%, respectively. The highly purified eLH, eFSH and respective antisera were provided by Dr. A. F. Parlow, National Hormone and Peptide Program (NHPP), Harbor-UCLA Research and Education Institute, Los Angeles, CA.

Plasma concentrations of E2 were determined by RIA using a protocol validated previously in bovine serum/plasma [81] and validated for equine plasma in our laboratory. Triplicate samples (300 μL) were extracted with 4 mL of HPLC grade methyl-tert-butyl ether (MTBE; Fisher Scientific, Hampton, NH) in 16 x 100 mm glass tubes for 1 minute using a multitube vortexer. Samples were then frozen in an -80° C ultra-low freezer for 20 minutes. The solvent fraction was decanted into corresponding 12 x 75 mm borosilicate glass tubes and dried at room temperature under industrial-grade nitrogen gas. The dried extract was rehydrated with 37° C 100 μL 1% BSA (1% BSA, 0.01% sodium azide, 0.01 *M* PO_4, and 0.9% NaCl; pH 7.2). The redissolved extracts and E2 standards (0.25, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 20.0, and 30.0 pg/tube) were incubated with 100 μL of estradiol-17β antisera (MP Biomedical, Santa Ana, CA, product #07-138216, 1:150,000 vol/vol dilution) for 2 hours on ice at 4° C. Following incubation, ^{125}I-estradiol-17β was added to each tube at a volume of 100 μL (MP

Biomedical, product #07-138228, adjusted to 5,000 cpm/tube). All tubes were incubated at 4° C for 20 hours. Bound and free E2 were separated by adding 0.5 mL of an ice cold (4° C) charcoal-dextran suspension and then centrifugation at 3500 rpm for 45 minutes at 4°C. The resulting supernatant was decanted into corresponding polypropylene tubes and counted in a gamma counter for 1 minute per tube. Cross-reactivities of this antibody have been reported as 100% for estradiol-17β, 6.5% for estriol, 5.2% for estradiol-17α, 0.6% for estrone, and <0.01% for androstenedione, cholesterol, aldosterone, progesterone, and testosterone [81]. The addition of known amounts of estradiol-17β to pooled equine plasma (2.5, 5.0, and 50.0 pg/mL) was recovered with an average percent recovery of added mass of 108%. Overall percent recovery for extraction was 92.3%. Intra- and inter-assay coefficients of variation were 8.4% and 7.1%, respectively, and sensitivity was 0.25 pg/mL in mare plasma.

Plasma estrone sulfate concentrations were determined by RIA performed by Dr. Scott Jaques at the Texas A&M University Veterinary Medical Diagnostic Laboratory in College Station, TX.

3.2.10 Statistical analysis

Temporal changes in peripheral concentrations of hormones and uterine edema were analyzed using a mixed model for repeated measures with main effects of treatment and day of treatment using JMP Pro 12.0.1 statistical software [SAS Institute, Cary, NC]. The model included random effects of day as the within subject factor, treatment as between-subject factor, and mare as the subject. Fixed effects were treatment, day of treatment, and the interaction of treatment x day. Repeated effect was day and the

subject was mare. If fixed effects were found to be significant ($P < 0.05$), a Tukey's HSD pair-wise comparison was performed. Analyses of ovulation frequency and frequency of behavioral estrus were analyzed via the Fisher Exact Test. Duration of behavioral estrus, interval to behavioral estrus, and the interval to ovulation were analyzed via one-way ANOVA. Main effects were deemed significant with $P \leq 0.05$ and a trend toward significance with $P \leq 0.10$.

3.3. Results

3.3.1 Uterine edema, teasing behavior and circulating E2

The primary objectives of this experiment were to assess pituitary and ovarian responses to GnRH and GnRH + E2 treatments. However, other secondary characteristics associated with treatments were also evaluated as follows.

3.3.1.1 Uterine edema

Similar to edema observed during the preliminary trial, a marked increase in uterine edema score was observed on Day 2 after treatment onset in mares in the GnRH + High E2 treatment, with their mean (± SEM) score of 2.33 ± 0.28 greater ($P = 0.0052$) than those in all other treatments (Figure 3.1). By Day 4, mean uterine edema scores in the GnRH (2.33 ± 0.28) and GnRH + Low E2 (2.50 ± 0.28) treatments were also increased. Edema scores remained greater ($P < 0.004$) in all GnRH- and GnRH + E2-treated groups compared to Controls through day 8 (Figure 3.1). Overall mean edema scores during the first 14-day GnRH pump/implant period for mares in the GnRH, GnRH + Low E2, and GnRH + High E2 groups (1.60 ± 0.12, 1.56 ± 0.12, and 1.77 ± 0.12, respectively) were greater ($P < 0.0001$) than those for Controls (0.58 ± 0.12). Observable edema dissipated within 48 hours following ovulation/implant-pump removal in treated groups. Ovarian-unresponsive mares in the GnRH + High E2 treatment group failed to maintain a measurable increase in uterine edema past Day 14 of treatment and did not reestablish edema scores greater than Controls following pump replacement on Day 14.

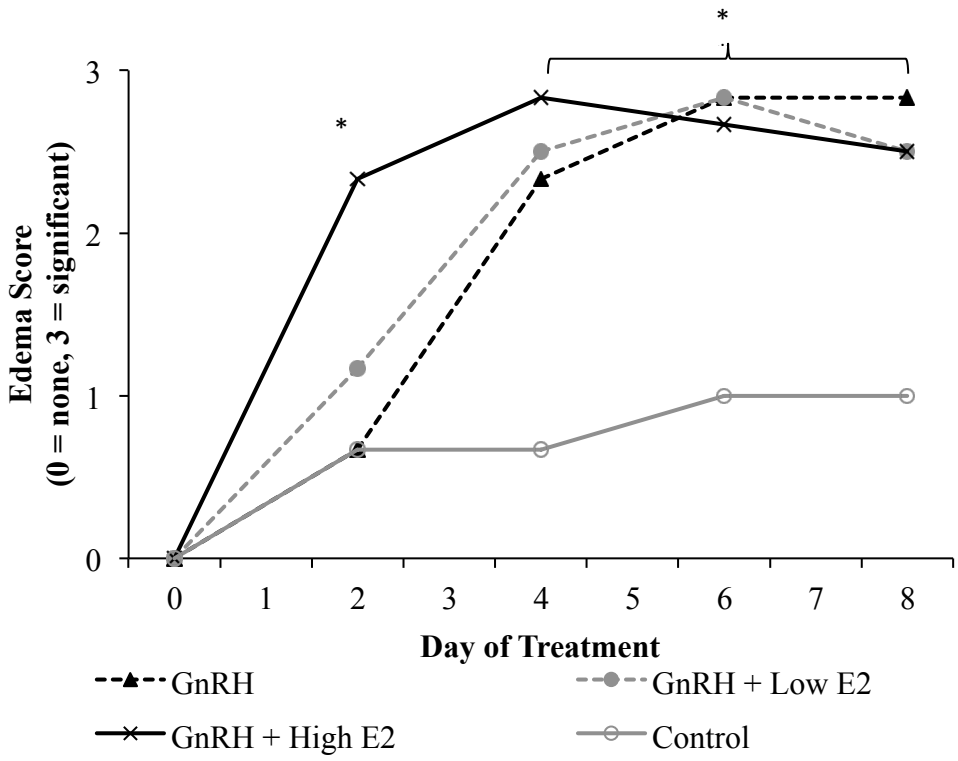

Figure 3.1. Uterine edema scores over the first 8 days of treatment. Edema scores range from 0 = no edema, 1 = slight edema, 2 = moderate edema, and 3 = normal maximal edema [78]. Mares in the GnRH + High E2 group had greater ($P < 0.01$) uterine edema by Day 2 compared to all other treatments. All GnRH-treated groups had greater ($P < 0.004$) uterine edema compared to Controls beginning on Day 4 and continuing through Day 10 of treatment. Values are least squares means with a pooled SEM of 0.28.

3.3.1.2. Teasing scores

Observed behavior on teasing with a stallion through the first 14 days of treatment is summarized in Table 3.1. All mares (6/6) in the GnRH only group displayed estrus (teasing score = 4) by Day 6 of treatment and all GnRH + High E2 mares (6/6) displayed estrus by Day 7, with all mares in the latter group maintaining a score of 4 through Day 12. The mean maximal teasing score for GnRH + Low E2 mares was 3.5 and was achieved in all mares by Day 6 of treatment. Three of the 6 Control mares displayed estrus, but the duration of behavioral estrus was significantly shorter (2.2 days; $P = 0.0042$) compared to all other treatments (≥ 7.3 days; Table 3.1). Despite the 3 Control mares displaying estrus, the observed largest average follicular diameter was markedly smaller (20.8 ± 2.8 mm; $P < 0.05$) compared to both the GnRH and GnRH + Low E2 groups (37.0 ±2.8 mm, 36.1 ±2.8 mm, respectively), in which 6/6 mares in each group were induced to ovulate.

	Control	GnRH	GnRH + Low E2	GnRH + High E2
Interval to Behavioral Estrus (days)	9.0 ± 1.5^{a}	4.8 ± 1.5^{b}	4.0 ± 1.5^{b}	4.5 ± 1.5^{b}
No.	$3/6^{a}$	$6/6^{b}$	$6/6^{b}$	$6/6^{b}$
Duration of Estrus (days)	2.2 ± 1.2^{a}	7.3 ± 1.2^{b}	9.2 ± 1.2^{b}	7.7 ± 1.2^{b}
Frequency of Ovulation	$0/6^{c}$	$6/6^{d}$	$6/6^{d}$	$2/6^{c,d}$
Largest Average Follicle (mm)	20.8 ± 2.8^{c}	37.0 ± 2.8^{d}	36.1 ± 2.8^{d}	25.9 ± 2.8^{c}

Table 3.1. Average interval to full behavioral estrus (teasing score = 4), frequency of full behavioral estrus, duration of full behavioral estrus, frequency of ovulation, and largest average follicle diameter over the first 14 days of treatment. Averages and frequencies with different superscripts differ ($^{a,b}P \leq 0.05$); ($^{c,d}P < 0.01$).

3.3.1.3. Plasma concentrations of E2

Because of rapid changes in follicle development, ovarian steroidogenesis, and occurrence of ovulation in response to GnRH and GnRH + E2 treatments, the first 7 days of the experiment were used initially to assess potential treatment-induced differences in circulating E2. Surprisingly, and in spite of clear changes in uterine edema scores, mean plasma concentrations of E2 did not differ ($P > 0.1$) among groups during the first 7 days of treatment (Figure 3.2).

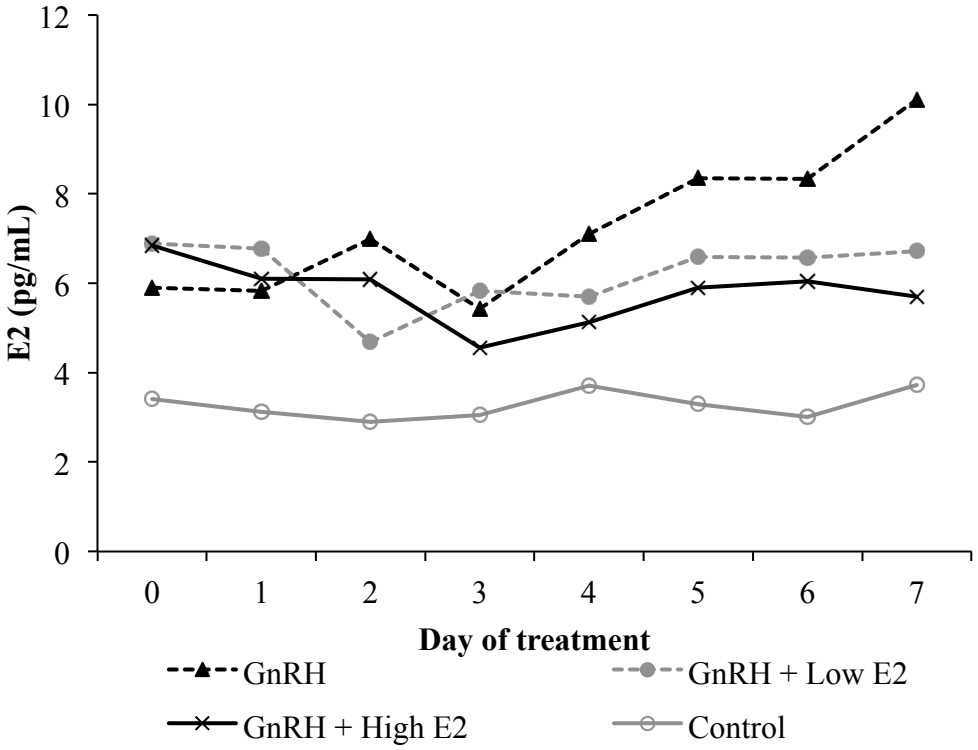

Figure 3.2. Least squares mean concentrations of peripheral E2 over the first 7 days of treatment. Mean concentrations did not differ. Pooled SEM = 1.55 pg/mL.

In ovarian-responsive mares, defined as developing a 35-mm follicle followed by induced ovulation with hCG, mean peripheral E2 concentrations were normalized to the day of induced ovulation (Day 0, Figure 3.3). During this period, responsive mares (6/6) in the GnRH group had greater concentrations of E2 (9.89 ± 1.37 pg/mL, $P = 0.02$) compared to Control mares (3.28 ± 1.37 pg/mL), with maximum concentrations (≥ 10.9 pg/mL) beginning 3 days prior to ovulation greater ($P < 0.05$) than those (≤ 3.7 pg/mL) observed in Control mares during the same period. Responsive GnRH + Low E2 mares (6/6) also exhibited greater circulating E2 (11.5 pg/mL, $P = 0.04$) on Day 2 before ovulation compared to unresponsive Control mares (3.2 pg/mL), whereas the 2 responsive GnRH + High E2 tended to be greater (6.3 pg/mL, $P < 0.1$) than Control mares on Day 2. Mean circulating E2 prior to ovulation did not differ among responsive GnRH, GnRH + Low E2, or GnRH + High E2 mares (Figure 3.3).

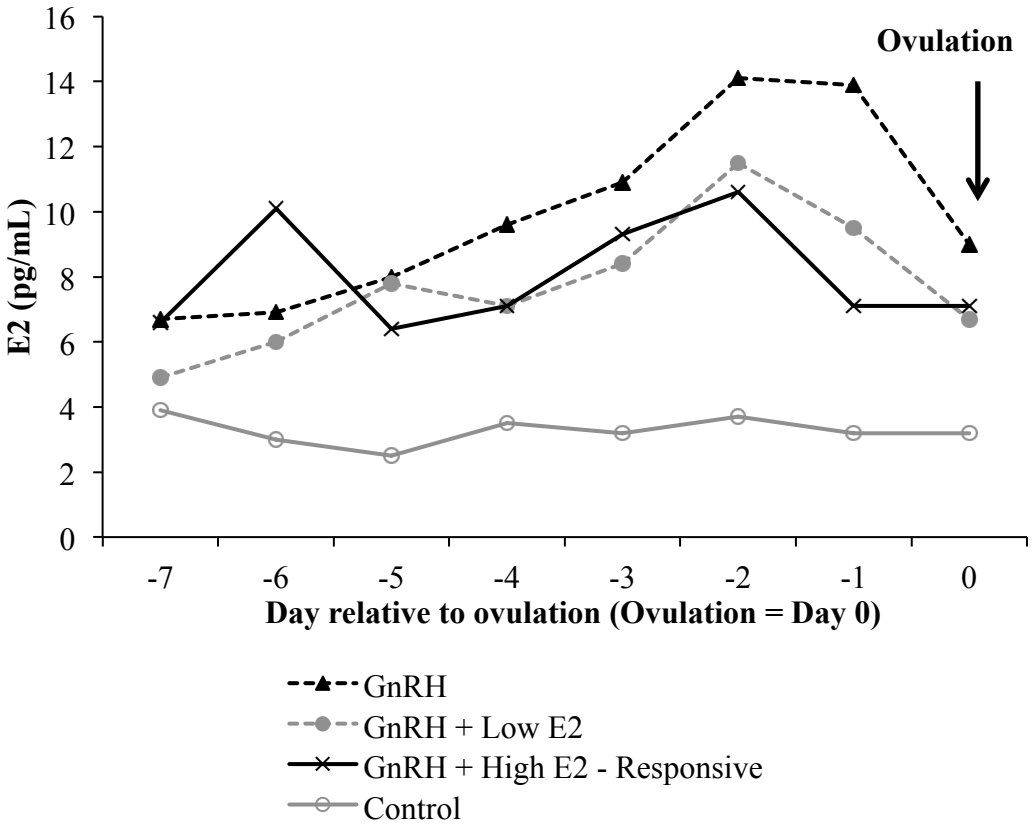

Figure 3.3. Least squares mean concentrations of peripheral E2 normalized to the day of ovulation in responsive mares within each treatment, and in all Controls, which did not ovulate (no normalization). Responsive GnRH mares (6/6) had greater mean E2 ($P <$ 0.05) beginning 3 days before ovulation compared to unresponsive Controls (6/6). Responsive GnRH + Low E2 mares (6/6) had greater ($P = 0.04$) mean E2 on Day 2 before ovulation compared to unresponsive Controls. Responsive GnRH + High E2 mares (2/6) tended to have greater mean E2 ($P < 0.10$) on Day 2 when compared to unresponsive Controls. Pooled SEM for GnRH, GnRH + Low E2, and Controls was 1.63 pg/mL, whereas pooled SEM for GnRH + High E2 was 2.83 pg/mL.

3.3.1.4. Plasma concentrations of estrone sulfate

Previous evidence suggests that exogenous E2 may be efficiently converted into estrogen metabolites such as estrone sulfate in mares [82]. In the present study, peripheral estrone sulfate paralleled circulating E2. The effects of treatment and treatment by day were significant ($P = 0.02$ and $P < 0.0001$, respectively; Figure 3.4). Unexpectedly, GnRH + High E2 mares had elevated ($P = 0.03$) baseline estrone sulfate (Day 0; 403.7 ± 91.7 pg/mL) compared to GnRH + Low E2 mares, but did not differ from GnRH or Control mares (257.4 ± 91.7 pg/mL and 277.6 ± 91.7 pg/mL, respectively). On Day 3 of treatment, estrone sulfate in GnRH + Low E2 mares tended ($P = 0.09$; 607.4 ± 91.7 pg/mL) to be greater than in GnRH alone mares (415.5 ± 91.7 pg/mL), but was not different than in GnRH + High E2 mares (516.1 ± 91.7 pg/mL). By Day 6 and coinciding with the development of dominant follicles in responsive mares, both GnRH and GnRH + Low E2 groups had greater ($P \leq 0.01$; 837.6 ± 91.7 pg/mL and 1006.0 ± 91.7 pg/mL, respectively) plasma estrone sulfate than Control mares (314.5 ± 91.7 pg/mL) and tended ($P \leq 0.09$) to be greater than GnRH + High E2 mares (504.2 ± 91.7 pg/mL).

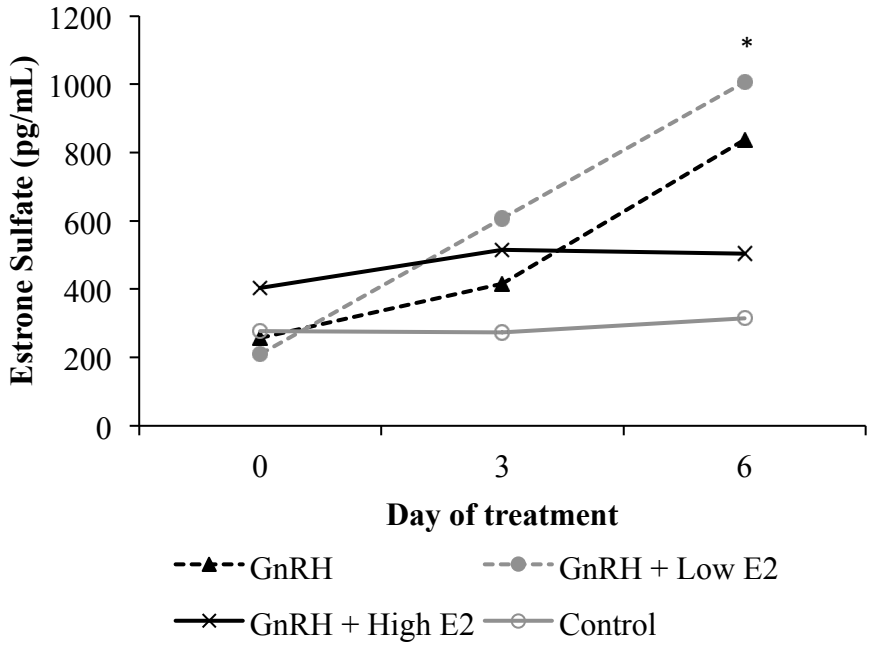

Figure 3.4. Mean plasma estrone sulfate during the first 6 days of treatment. The GnRH + High E2 mares had an elevated ($P = 0.03$; 403.7 pg/mL) baseline on Day 0 compared to GnRH + Low E2 mares (209.3 pg/mL). The GnRH + Low E2 group tended ($P \leq 0.09$) to have greater mean estrone sulfate on Day 3 compared to GnRH and Control mares. Both the GnRH alone and GnRH + Low E2 groups had increased ($P < 0.01$) mean estrone sulfate than the Control group and tended to have greater concentrations ($P = 0.09$) than GnRH + High E2 mares. Pooled SEM was 91.7 pg/mL for all groups.

3.3.2 Pituitary responses

3.3.2.1 Plasma concentrations of LH

Mean plasma concentrations of LH are shown in Figure 3.5. Mean LH in GnRH, GnRH + Low E2, and GnRH + High E2 treatment groups did not differ ($P > 0.10$, Figure 3.5A); therefore, values for these 3 groups were pooled. Beginning on Day 2 and continuing through Day 7, mean concentrations of LH increased and were greater ($P < 0.01$) in GnRH-treated mares than in Controls (Figure 3.5B).

Importantly, mean LH in ovarian-responsive (0.72 ± 0.07 ng/mL) and ovarian-unresponsive (0.93 ± 0.13 ng/mL) GnRH-treated mares did not differ until Day 13, coinciding with timing of induced ovulation and removal of osmotic pumps in the majority of responsive mares (Figure 3.6). By Day 16, majority of pumps (13/14) had been removed in the GnRH-treated, ovarian-responsive mares, and from this point until the end of the study on day 28 mean LH in ovarian-unresponsive mares remained above ($P < 0.03$) ovarian-responsive mares (Figure 3.6). In the ovarian-unresponsive (4/6) GnRH + High E2 mares (not shown), circulating concentrations of LH was greater ($P < 0.01$) than Controls (6/6 mares; 0.27 ± 0.11 ng/mL) beginning on Day 2 and continuing for the duration of the 28-day treatment period. A decline ($P < 0.05$) in concentrations of LH was observed in unresponsive GnRH + High E2 mares before replacement of the first GnRH osmotic pump on day 14, but values rebounded by day 18 (Figure 3.6).

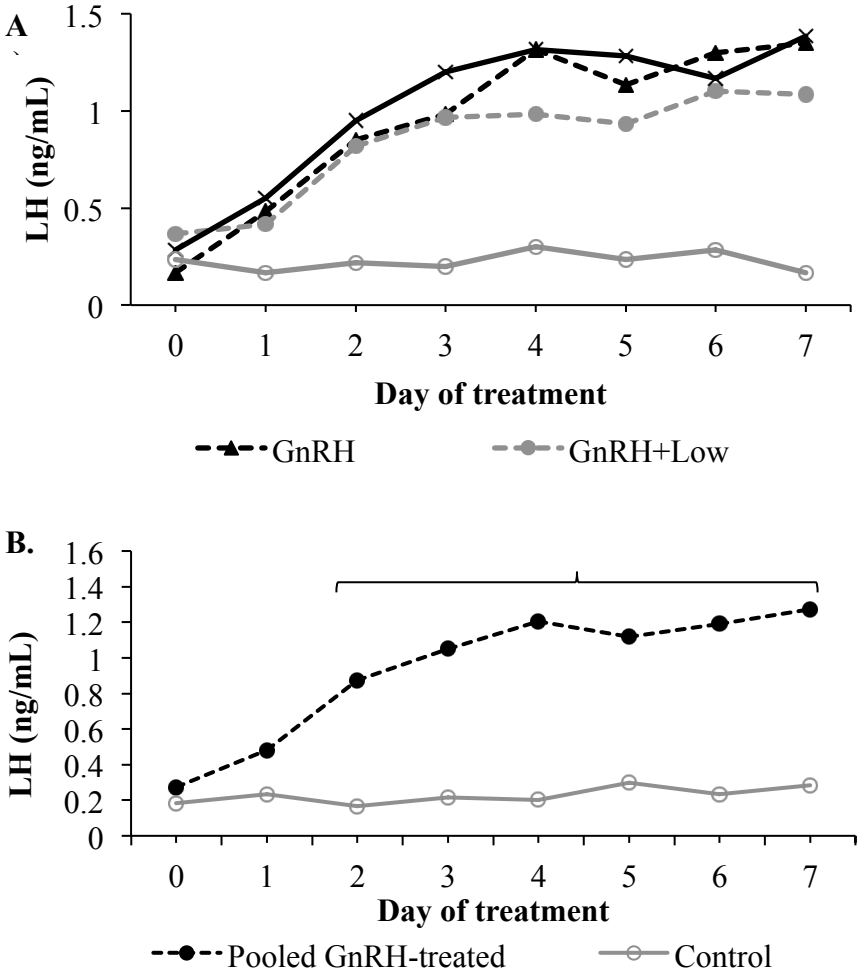

Figure 3.5. Plasma LH concentrations over initial 7 days of treatment. A.) Mean LH concentrations over first 7 days of treatment in all treatment groups (Pooled SEM = 0.6 ng/mL for all treatment groups). B.) Mean LH concentrations of GnRH- and GnRH + E2-treated mares did not differ ($P > 0.10$) and were therefore pooled for comparison to Controls. Pooled least squares mean concentrations of LH in GnRH- and GnRH + E2-treated mares (n = 18) and in control mares (n = 6) during the first 7 days after treatment onset. Concentrations of LH in GnRH-and GnRH + E2-treated mares were greater ($P < 0.01$) than Controls from Day 2 through 7. (Pooled SEM = 0.2 ng/mL).

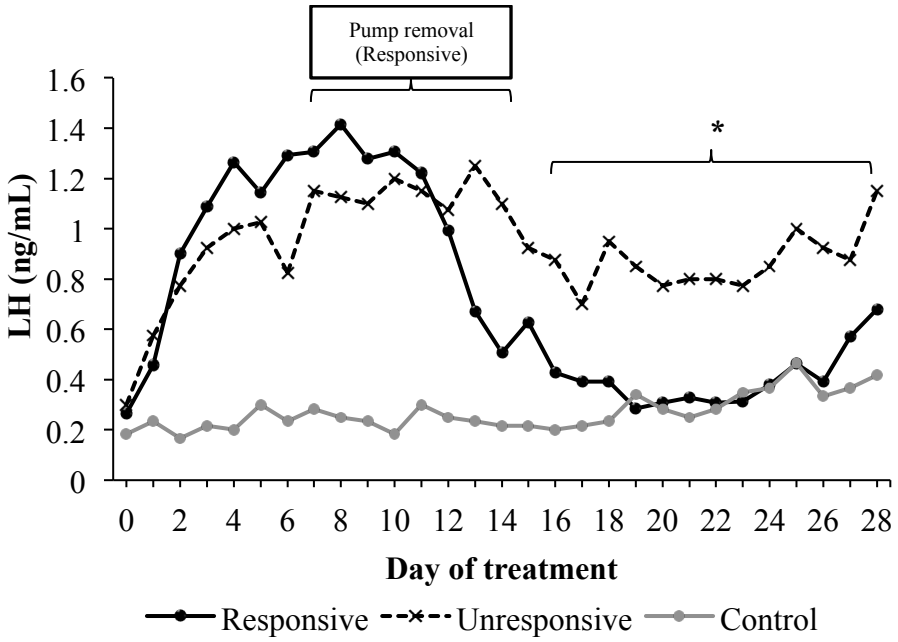

Figure 3.6. Least squares mean concentrations of LH in ovarian-responsive and ovarian-unresponsive GnRH-treated mares over the 28-day experiment. No differences were observed between ovarian-responsive and ovarian-unresponsive GnRH-treated mares until Day 13, coinciding with onset of ovulations and pump removal in responsive mares. Ovarian-unresponsive mares had persistently greater peripheral LH than both ovarian-responsive ($P \leq 0.03$) and Controls ($P \leq 0.05$) for the remainder of treatment. Pooled SEM = 0.2 ng/mL.

3.3.2.2 Plasma concentrations of FSH

As observed with circulating LH, mean concentrations of plasma FSH in mares in GnRH, GnRH + Low E2, and GnRH + High E2 treatment groups did not differ during the first 7 days following treatment initiation. Therefore, values for mares in these groups were pooled into a single GnRH-treated group for further analysis. Concentrations of FSH increased in GnRH-treated mares and reached a peak (8.4 ± 0.7 ng/mL; $P < 0.05$) 24 hours after treatment began relative to Controls (5.4 ± 1.2 ng/mL), regardless of ovarian responsiveness (Figure 3.7). Mean FSH concentrations and follicular development from representative mares from each treatment group are shown in Figure 3.8. The observed peak in FSH dissipated within 48 hours and mean concentrations of FSH returned to baseline or lower, depending on ovarian responsiveness, in all mares by Day 4 of treatment.

Similar to ovarian responsive mares treated with GnRH, the FSH peak for ovarian-unresponsive mares (4/6 GnRH + High E2 mares) was greater (10.35 ng/mL, $P = 0.03$) than Controls (5.37 ng/mL) at 24 hours after treatment onset (Figure 3.9). Following the initial peak, FSH returned to baseline (ovarian-unresponsive) or lower (ovarian-responsive) concentrations for the duration of the treatment period. Mean FSH concentrations in ovarian-unresponsive mares did not differ (6.42 ± 0.83 ng/mL, $P > 0.1$) from Control (5.56 ± 0.68 ng/mL) or ovarian-responsive mares (4.84 ± 0.36 ng/mL, Figure 3.9), following the initial peak.

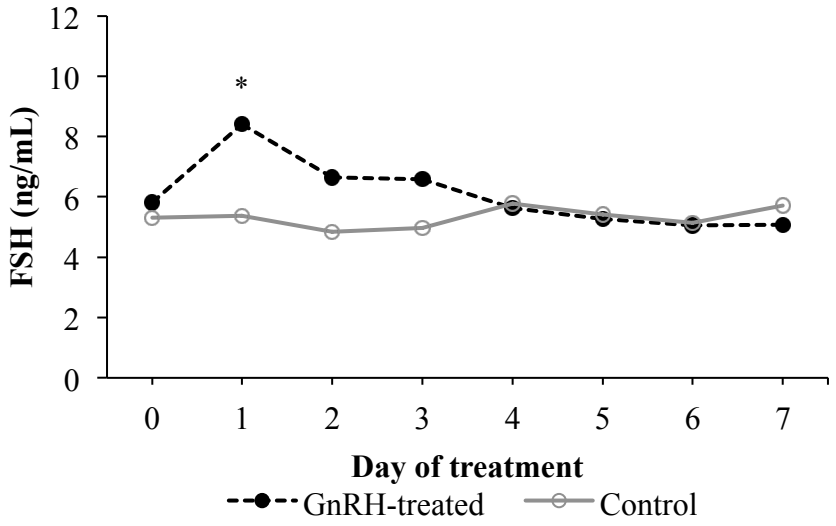

Figure 3.7. Pooled least squares mean concentrations of FSH for all GnRH- and GnRH + E2-treated mares (n = 18/18) and Control mares (n = 6/6) over the initial 7 days after treatment initiation. Pooled SEM = 0.8 ng/mL. (* $P < 0.05$).

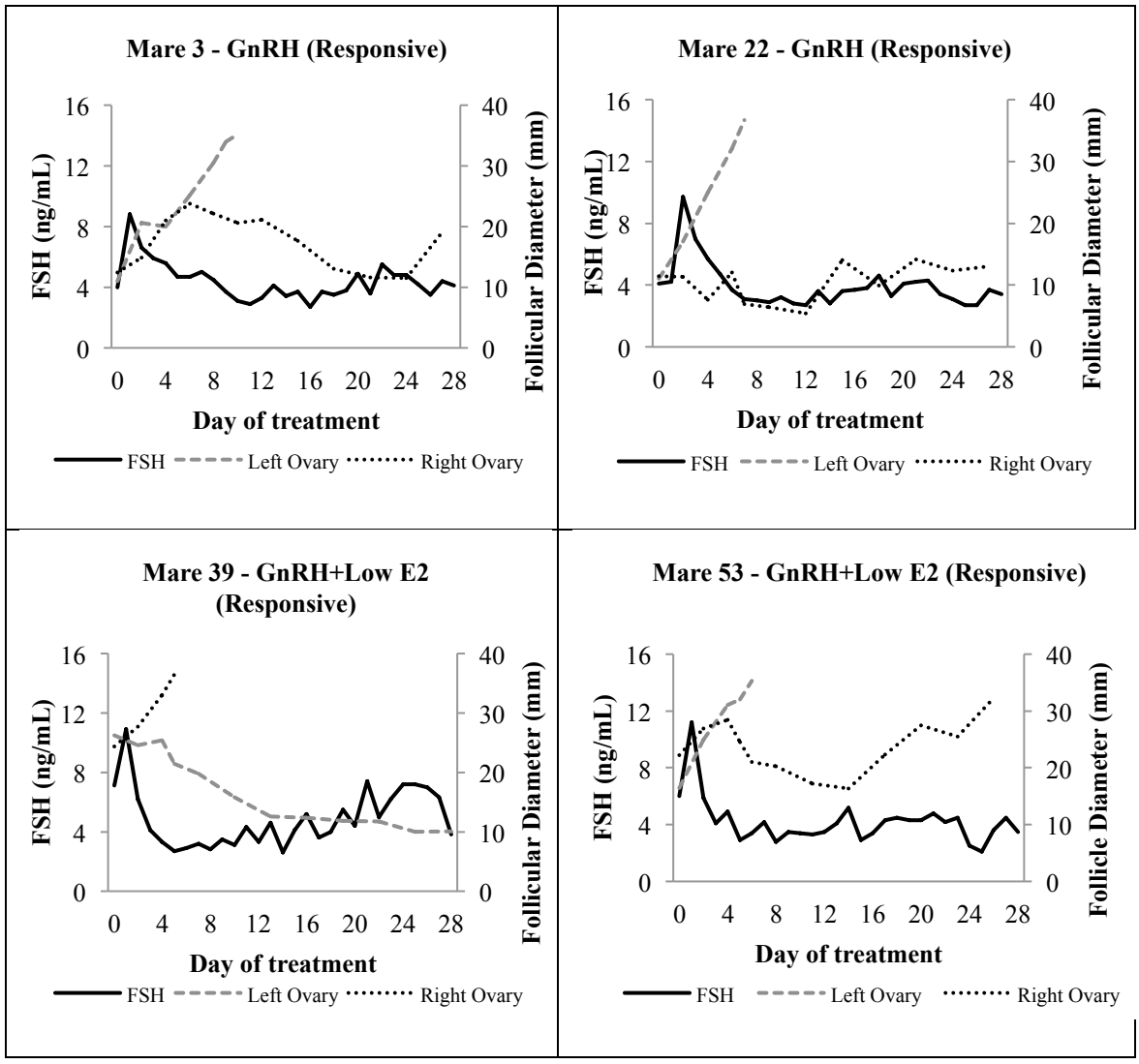

Figure 3.8: Individual profiles of FSH and follicular development during the 28-day treatment period for representative mares in each treatment group, including ovarian responsive and unresponsive individuals.

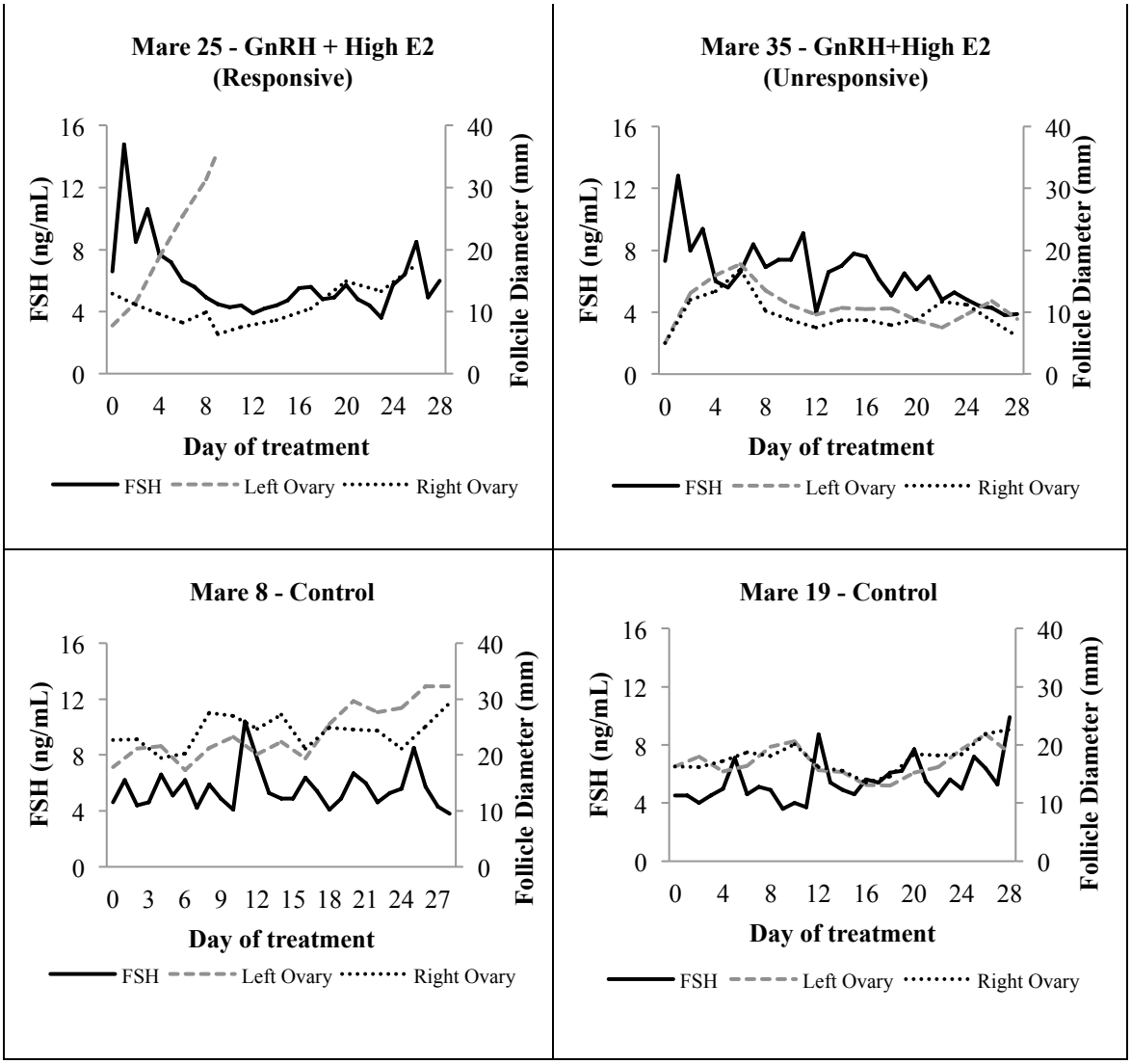

Figure 3.8 continued: Individual profiles of FSH and follicular development during the 28-day treatment period for representative mares in each treatment group, including ovarian responsive and unresponsive individuals.

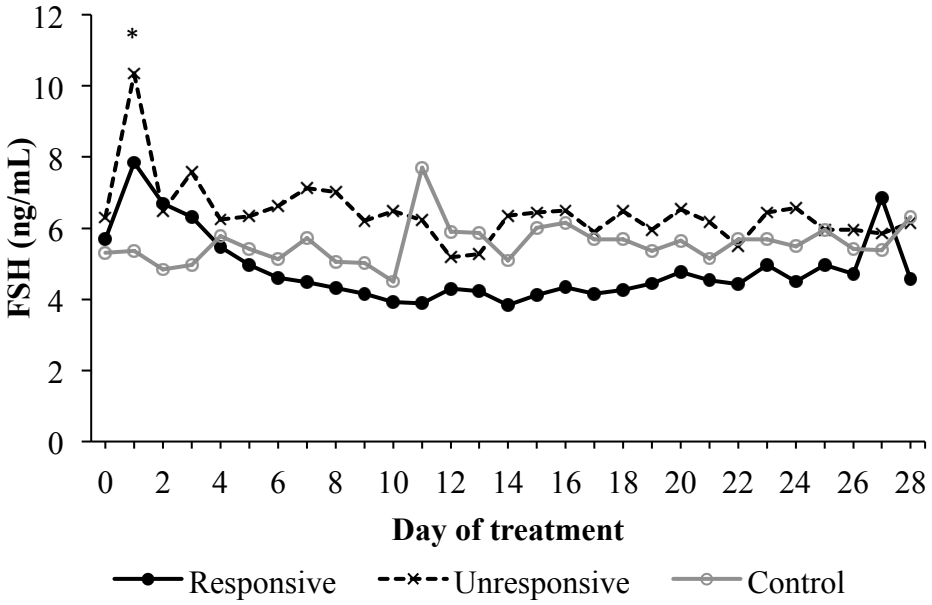

Figure 3.9. Least squares mean concentrations of FSH for GnRH- and GnRH + E2-treated, ovarian-responsive mares (12/12), unresponsive GnRH + High E2 mares (4/6), and Control mares (6/6) over the 28-day experiment. Peak concentrations of FSH at 24 hours after treatment onset for ovarian-unresponsive mares was greater (10.35 ng/mL, $P = 0.03$) than that of Controls (5.37 ng/mL). Pooled SEM for ovarian-responsive, ovarian-unresponsive, and Control mares were 0.8 ng/mL, 1.1 ng/mL, and 0.6 ng/mL, respectively.

3.3.2.3 GnRH Challenge

Mares that failed to develop a 35-mm follicle and thus were not treated with hCG to induce ovulation within the 28-day experimental period (n = 4/6 GnRH + High E2; n = 5/6 Controls) were evaluated further by administering a GnRH challenge to assess releasable pools of LH and FSH. One Control mare developed a 35-mm follicle by Day 28 of the experiment and was excluded from the GnRH challenge although she was not treated with hCG.

As presented above, mean concentrations of LH for ovarian-unresponsive GnRH + High E2 mares were greater than control mares at the end of the experiment. Thus, mean baseline (Time = 0 min; 1.23 ± 0.14 ng/mL) concentrations at onset of the GnRH challenge in the former group were greater ($P < 0.01$) than Controls (0.2 ± 0.12 ng/mL) as shown in Figure 3.10A and remained greater ($P = 0.0009$; 1.33 ± 0.09 ng/mL) than Control mares (0.64 ± 0.08 ng/mL) throughout the post-GnRH sampling period. Because of the differing baseline, mean concentrations were transformed to a percent of Time -15 min values as presented in Figure 3.10B. Beginning 15 minutes after bolus GnRH injection, percent change in mean LH in Controls increased ($P = 0.01$) and remained elevated above GnRH + High E2 mares and the pre-treatment mean until the end of sampling at 240 minutes. Treatment with GnRH had no effect on circulating concentrations of LH in the GnRH + High E2 mares (Figures 3.10A and Figure 3.10B).

Unlike mean LH, baseline concentrations of FSH in GnRH + High E2 and Control mares did not differ. Treatment with GnRH at Time 0 resulted in increased concentrations ($\geq$ 7.86 ng/mL; $P < 0.0001$) of FSH in Controls compared to GnRH + High E2 mares (3.76 ng/mL) beginning 15 minutes after and continuing through 240 minutes. As noted earlier, mean concentrations of FSH did not change in GnRH + High E2 mares after GnRH treatment (Figure 3.11).

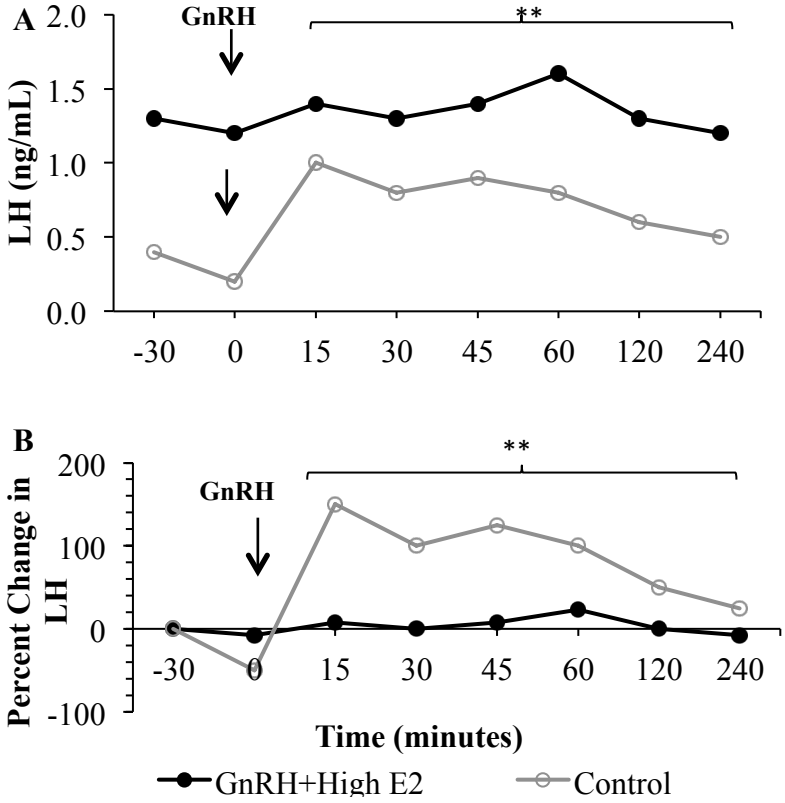

Figure 3.10. Plasma LH response to bolus, intravenous injection of 1 mg GnRH in ovarian-unresponsive GnRH + High E2 mares (n = 4/6) and Control mares (n = 5/6). A.) Least squares mean LH beginning 30 min before bolus GnRH injection (Time = 0) and lasting until 240 minutes post-injection. B.) Least squares mean concentrations of LH presented as a percent of Time -15 minute values for ovarian-unresponsive GnRH + High E2 mares (4/6) and Control mares (5/6) due to elevated ($P < 0.01$) baseline in GnRH + High E2 mares. Control mares tended to have a greater percent change ($P < 0.07$) in mean LH compared to Controls beginning 15 minutes after GnRH injection and lasting until 240 minutes post-injection. Mean values in GnRH + High E2 mares did not differ from baseline after GnRH challenge. Pooled SEM for GnRH + High E2 mares and Control mares were 0.12 ng/mL and 0.14 ng/mL, respectively.

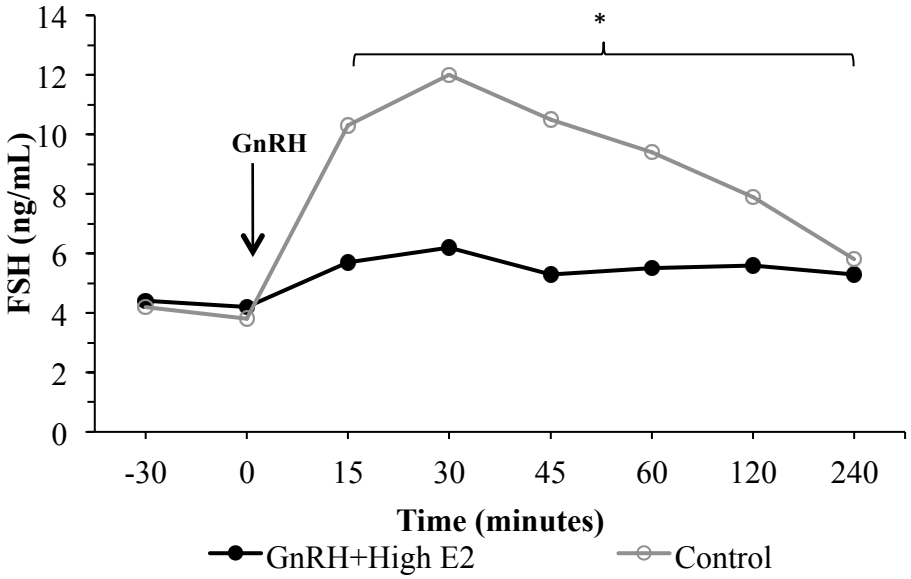

Figure 3.11. Least squares mean concentrations of FSH beginning 30 min before and continuing for 240 minutes after bolus intravenous injection of GnRH (Time 0) in Control mares and ovarian-unresponsive mares in the GnRH + High E2 group. Concentrations of FSH in Control mares treated with GnRH were greater ($P < 0.01$) than the pre-treatment baseline and greater than the GnRH + High E2 group which did not change from baseline. Pooled SEM for GnRH + High E2 and Control mares was 1.25 ng/mL and 1.12 ng/mL, respectively.

3.3.3 Ovarian responses

3.3.3.1 Follicular development and ovulation

Ovarian characteristics are presented in Table 3.2. Baseline follicular diameter was not different amongst treatment group ($P > 0.1$; data not shown). None of the Control mares (0/6) were induced to ovulate within the 28-day treatment period and only 1 developed a 35-mm follicle, which occurred on Day 28. However, within the first 15 days of treatment onset, 5/6 GnRH and 6/6 GnRH + Low E2 mares had developed a 35-mm follicle and were induced to ovulate. Only 2/6 GnRH + High E2 mares developed a 35-mm follicle, both by Day 9, and were induced to ovulate by Day 15 of treatment. To facilitate statistical analysis, mares that did not ovulate within the 28-day treatment window were assigned an arbitrary interval to ovulation of 28 days. Frequency of development of a 35-mm follicle and ovulation were greater in GnRH and GnRH + Low E2 than in Controls and intervals to these end-points were markedly reduced compared to Controls (Table 3.2). The lifespan of induced corpora lutea and average interval to the next pre-ovulatory follicle was not different among ovulatory mares regardless of treatment (data not shown).

	Control	GnRH	GnRH+Low E2	GnRH+High E2
Interval to 35 mm follicle, d	28.0	11.7	8.7	21.7
No. mares	1/6[a]	6/6[b]	6/6[b]	2/6[a,b]
Interval to ovulation, d	28	13.7 ± 7.2	10.7 ± 2.9	23.7± 9.8
No. mares	0/6[c]	6/6[d]	6/6[d]	2/6[c,d]
Avg. date First ovulation	N/A	Feb. 22	Feb.21	March 3

Table 3.2. Average interval to 35-mm follicular development, interval to ovulation, number of ovulatory mares per treatment, and average date to first ovulation in groups with responsive mares. Means within rows with different superscripts differ ($^{a,b}P < 0.015$), ($^{c,d}P < 0.002$)

3.3.3.2 Luteal progesterone

Post-ovulatory concentrations of plasma progesterone did not differ among ovarian-responsive mares in GnRH (6/6), GnRH + Low E2 (6/6) and GnRH + High E2 (2/6) treatment groups (Figure 3.13; $P = 0.11$). Average functional CL lifespan, defined as concentrations of progesterone remaining above 1.0 ng/mL, did not differ among GnRH (14.8 days), GnRH + Low E2 (14.0 days), and GnRH + High E2 (13.5 days) treatment groups.

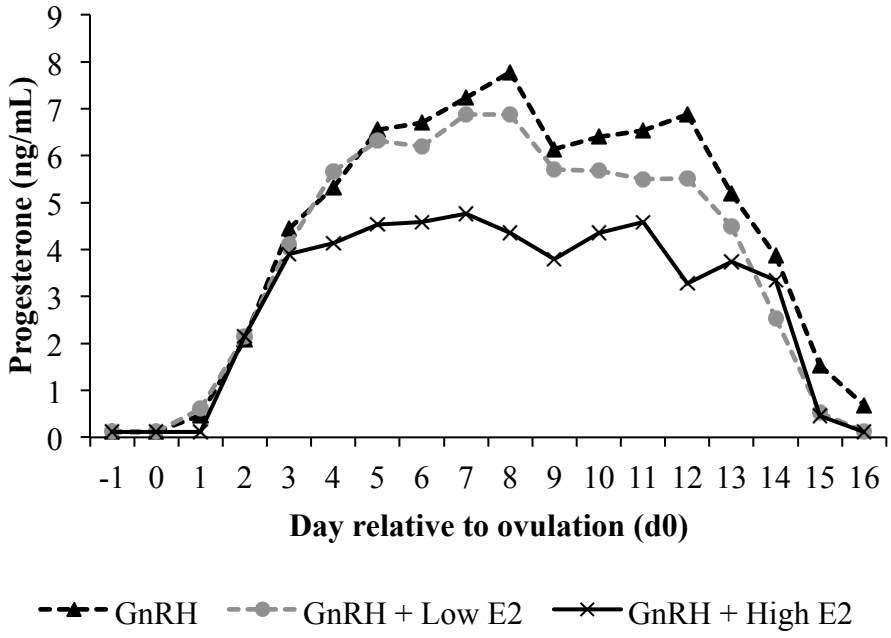

Figure 3.12. Mean peripheral P4 relative to day of ovulation (Day 0) in ovulatory GnRH alone (6/6), GnRH + Low E2 (6/6) and GnRH + High E2 (2/6) mares. Pooled SEM for GnRH alone and GnRH + Low E2 is 0.58 ng/mL, pooled SEM for GnRH + High E2 is 1.01 ng/mL.

3.4. Discussion

Based on findings in the current experiment, continuous treatment of winter anovulatory mares with E2 using a subcutaneous estradiol implant, designed to create either a relatively low (1/4 implant) or high (full implant) estrogenic environment, did not enhance gonadotropic responses to continuous treatment with GnRH. Similarly, there was no enhancement of ovarian responses, although this interpretation is based on a relatively small number of observations for this type of variable. The E2 source utilized in this study (Compudose implant), when placed subcutaneously into the ear of cattle, creates mean E2 concentrations of approximately 100 pg/mL over a long duration [83] and creates growth-promoting effects in that species for at least 200 days. Although the relative efficacy of these implants for elevating plasma E2 in mares has not been studied previously, the basic concepts associated with elution of steroid hormones from Silastic rubber implants administered subcutaneously has been widely reported in livestock species [84-87]. In our preliminary experiments, both the 1/4-sized and full implants alone resulted in significant to extensive uterine edema in all mares examined within 24-48 hours after implant insertion. However, despite a similar observation in the current experiment, peripheral plasma E2 was not increased in a dose-related fashion relative to Controls or to mares treated with only GnRH.

It has been well established that mares quickly convert circulating E2 into conjugated estrogens, including estrone sulfate, with plasma concentrations of conjugates measuring 100-fold greater than plasma E2 [88]. The relative efficiency of this conversion, especially in response to exogenous treatment with E2, has not been

well-defined. A recent study that evaluated the effects of varying doses (2.5 mg to 10 mg) of estradiol benzoate in cycling mares demonstrated that mean concentrations of circulating E2 never exceeded 14 pg/mL [82] and thus were not dose-related. To the contrary, the increase in estrone sulfate ranged from 200 to 600 pg/mL, and was dose-dependent [82], suggesting rapid conversion of exogenous E2 to a metabolic conjugate [89]. In the current experiment, we also assayed a selected number of samples for estrone sulfate using RIA. Estrone sulfate in these samples did not rapidly increase as expected in the GnRH + E2 treatments after treatment onset. However, estrone sulfate concentrations rose markedly by Day 6 in GnRH and GnRH + Low E2 mares, coinciding with the development of dominant follicles in these responsive mares. Though the conversion of estrone sulfate occurs as previously described [82,83,88,89], it was not associated with the onset of E2 treatment. However, the E2 eluted from the implants could have been converted immediately into metabolites other than estrone sulfate, such as estrone, equilin, estrone-3-glucuronide, or estradiol-3-sulfate [88], accounting for the marked uterine edema observed by Day 2 of treatment in the mares receiving the full E2 implant. Unfortunately, peripheral E2 metabolites other than estrone sulfate were not measured in this experiment. It is possible that the Compudose E2 implants may not effectively stimulate E2 above basal concentrations in the winter anovulatory mare and, therefore, the conversion into estrone sulfate is not discernable until a dominant follicle is present.

Similar to previous studies utilizing subcutaneous osmotic pumps to deliver native GnRH to winter anovulatory mares, continuous infusion stimulated increased

secretion of peripheral LH within 48 hours after treatment began [58,59]. Circulating concentrations of LH remained well above control values until pump removal following confirmed ovulation or at the end of the 28-day treatment. Both maximal and mean concentrations of LH were unrelated to ovarian responsiveness or baseline follicular diameter in the current study (data not shown), which is similar to previous observations reported from our laboratory [59]. The majority of GnRH-treated mares had developed a 35-mm follicle and were induced to ovulate within the first 15 days after treatment onset and all but one responsive mare (GnRH alone) had ovulated by the first week in March, regardless of E2 treatment. The timing of and intervals to first ovulation in this study correspond to those reported in previous work involving the use of native GnRH [55-57,80]. Although only 2 of 6 mares in the GnRH + High E2 group developed a 35-mm follicle and were induced to ovulate, the lack of response in the remaining mares (4/6) could not be related to any observed differences in secretion of LH or FSH, or any other feature that would differentiate them from the 10-15% of winter anovulatory mares that historically fail to respond to similar treatments [58-60,71]. In a previous study reported from this laboratory, twice daily injections of E2 in corn oil alone failed to increase mean circulating LH compared to controls during a 14-day treatment period in winter anovulatory mares [71]. However, twice daily E2 injections combined with continuous GnRH did enhance (0.5 to 0.8 ng/mL increases; $P < 0.05$) pituitary secretion of LH compared to continuous GnRH treatment only. Unfortunately, circulating E2 was not measured in that study. Therefore, the lack of a dose-related increase in either plasma E2 or estrone sulfate in the current study in response to a 1/4- or full-size Compudose

implant suggests that the implants failed to elute expected amounts of E2. As a result, neither plasma values of E2 nor estrone sulfate exceeded those expected and observed in association with the dominant follicles that developed in the majority (14/18) of GnRH and GnRH + E2-treated mares.

The equine pituitary has a limited number of monohormonal gonadotropes for either LH or FSH (9.7% during the winter anovulatory period, 22.5% during the breeding season), while the majority of gonadotropes are bihormonal [90]. Though not considered the limiting factor in winter anovulation [12,91], it was expected that FSH-secreting gonadotropes would respond similarly to LH-secreting gonadotropes and lead to a comparable pattern of secretion in response to continuous infusion of GnRH until development of a large, deviated follicle. Instead, FSH exhibited an acute peak approximately 24 hour after treatment onset and then declined abruptly. Acute increases in FSH due to continuous infusion of GnRH via an indwelling catheter have previously been reported followed by a subsequent decline within 5 to 7 days, depending on dose [55]. The rapid decline was attributed to possible increasing concentrations of intra-follicular E2 and inhibin, since all GnRH-treated mares in that experiment ovulated by Day 15 [55]. However, authors were unable to confirm the same type of response in a second experiment [56]. Similar to changes in LH, concentrations of FSH were much greater in response to hourly i.v. injections of GnRH [55,56] when compared to subcutaneous treatment as shown in the study herein. In the current study, FSH declined to baseline (ovarian-unresponsive mares) or below (ovarian-responsive mares) by Day 4 of treatment and did not rebound while pumps remained *in situ*. Since the majority of

responsive mares had developed a 35-mm follicle well before Day 15 (93%), the decline in FSH in these mares could be attributed to inhibitory feedback of inhibin from the large follicle, similar to that suggested by Johnson et al. [55]. However, the consistency and timing of this response, especially in unresponsive mares, is more in keeping with differentially-controlled desensitization of FSHβ synthesis and secretion as compared to LH.

During the luteal phase in cycling mares, continuous infusion of GnRH has been shown to create rapid, episodic release of LH similar to that seen during the follicular phase [58]. While this has not been demonstrated to occur in anovulatory mares, the continuous mode of GnRH exposure may mimic the rapid, follicular phase pulses (1 pulse/hour) of GnRH known to favor LH synthesis and release [2]. In contrast, a slow frequency of GnRH pulses (1 pulse/3-4 hours) favors FSH synthesis and release [2]. Therefore, the continuous subcutaneous infusion of GnRH as described here and previously, although not physiologically optimal, may favor synthesis and secretion of LH while maximally down-regulating FSH synthesis and secretion. In addition, the initial stimulation with exogenous GnRH may exhaust the releasable pools of FSH already existent within the pituitary. The initial stimulation by GnRH may be sufficient for immediate FSH secretion, but may not stimulate transcription necessary for additional FSH synthesis. In sheep, it has been reported that a greater proportion of releasable FSH is released per day and is more closely related to the rate of synthesis than is LH [92,93]. Thus, Chappel et al [92] suggested it may be easier to exhaust the releasable pool of FSH than LH in response to repeated GnRH stimulation. If this holds

true in mares, it is possible that the initial observed peak in FSH reflects the releasable pool of FSH existent within the pituitary at the time of treatment onset, which becomes rapidly depleted. Once the existent pool is depleted, further GnRH stimulation causes immediate but indiscernible release of FSH without observable increases in the baseline in unresponsive mares.

Because ovarian-unresponsive mares (Controls n= 5/6; GnRH + High E2 n = 4/6) had not ovulated or resumed ovarian cyclicity during the 28-day experiment, we used them to contrast total releasable pools of LH and FSH in mares either treated (GnRH + High E2) or untreated with GnRH during that period. Modest but measurable increases in both gonadotropins relative to pre-treatment baselines were observed in Control mares but not in GnRH + High E2 mares. As noted previously, the pretreatment baseline for LH, but not FSH, was elevated in the GnRH-treated mares compared to Controls. Collectively, these observations suggest that both LH- and FSH-secreting gonadotropes had been maximally stimulated with continuous GnRH treatment and were unable to respond further. The basis of this unresponsiveness, as discussed above, could differ between the two cell types but, due to the limited population of monohormonal gonadotropes, is likely related to either GnRH receptor down-regulation and/or depletion of releasable pools as a result of the 28-day treatment. In contrast, Control mares had measurable but modest increases in LH release in keeping with near depletion of this gonadotropin in seasonally anovulatory mares during the winter [4,12]. However, release of FSH in response to GnRH challenge was much more substantial than for LH and

agrees with the concept that FSH synthesis and release is not significantly hindered during the anovulatory season [12].

As indicated earlier, follicular responses in this study were unrelated to baseline follicular diameters, similar to that observed in our previous report [59]. Given the patterns of LH and FSH release observed in response to GnRH, there was no basis to attribute lack of ovarian responsiveness to limitations in secretion of LH. With respect to FSH, acute changes in response to GnRH were essentially identical across treatments. However, once the opportunity for follicle selection had passed in connection with the initial FSH peak, FSH secretion patterns were unremarkable in unresponsive mares for the balance of treatment and additional peaks of FSH in association with growth of a dominant follicle were not observed. Therefore, we remain unable to explain the basis of the 10-15% ovarian unresponsiveness observed herein and in previous studies reported from our laboratory and others [57-60,71].

However, one factor potentially regulating the follicular response to GnRH is the ovarian intra-follicular environment itself. Deviation is said to occur when the future dominant follicle of mares is approximately 23 mm in diameter [94,95] and as FSH is declining [96]. Recent studies have pointed to IGF-1 as being a sensitizer to the declining FSH surge [97]. Supporting this theory, IGF-1 concentrations increase 1 day prior to deviation in ovulatory follicular waves [98] and regulate the production of inhibin-A and activin-A [99]. When subordinate follicles were injected with pharmacologic doses of IGF-1, they become dominant follicles in a majority of mares [99,100]. Conversely, dominant follicles injected with IGF binding proteins ceased

follicular growth and follicles soon became atretic [101]. In the current study, the four ovarian-unresponsive, GnRH + High E2-treated mares began a follicular wave within 48 hours of the FSH peak, but follicle diameters did not exceed 25 mm and regressed. Thus, we speculate that ovarian-unresponsive mares may have inadequate intra-follicular IGF-1 concentrations or greater concentrations of IGF binding proteins, thereby hindering sensitivity to FSH and preventing further follicular growth and ovulatory responsiveness.

Based off these findings, future investigations regarding the intra-follicular environment and its feedback on the pituitary of responsive and unresponsive mares during GnRH infusion could contribute to our understanding of this phenomenon. Regarding the E2 implants used simultaneously with native GnRH infusion in the current study, investigation into the circulating concentrations of metabolites other than estrone sulfate would be valuable to further ascertain the effectiveness of the implants in the equine as it relates to follicular response.

IV. CONCLUSIONS

The study herein furthers the understanding of the pituitary-ovarian effects of continuous infusion of subcutaneous, native GnRH in the winter anovulatory mare. In a previous experiment reported from this laboratory [71], twice daily intramuscular injections of E2 enhanced the response of winter anovulatory mares to continuous GnRH treatment over a 14-day period. In the current study, injected E2 was replaced by the continuous presence of either a small (1/4-size) or large (full-size) E2-containing implant (Compudose®). Based on the results of this experiment, neither implant enhanced pituitary responses to GnRH. This is in spite of the fact that mares exposed to a full implant exhibited normal, maximal uterine edema within 48 hours of treatment onset. However, mean E2 concentrations did not exceed 22 pg/mL, and both plasma E2 and estrone sulfate concentrations mirrored increases in E2 typically expected to occur in association with a large, dominant follicle, such as occurred in this study. Taken together, and in spite of the presence of a reliable estrogenic biomarker (uterine edema), these data question the effectiveness of Silastic implants such as those used here to achieve graded increases in E2 dose. However, it cannot be ignored that E2 eluted from the E2 implant could be metabolized immediately into an active metabolite other than estrone sulfate. Unfortunately, no other metabolites were assayed for in the current study.

In the current experiment, and similar to that reported previously from this laboratory [58,59], mean LH did not differ between ovarian-responsive (developed a 35-mm follicle and were induced to ovulate) and ovarian-unresponsive mares. Although not

examined previously, the response of FSH-secreting gonadotropes to continuous, subcutaneous treatment with native GnRH did not mimic that of LH. Instead, an acute increase in plasma FSH that peaked within 48 hours, then declined to baseline within 4 days, was observed. This pattern, although commonly observed in response to the feedback effects of estradiol and other components of a large, dominant follicle, seemed unrelated to such an explanation in the current study. All mares, regardless of whether they developed a large follicle or not, had the same FSH secretion pattern in response to GnRH treatment. Therefore, it appears that the continuous exposure of FSH-secreting gonadotropes to native GnRH, at the rate administered in this study, results in down-regulation of the GnRH receptor as it relates to FSH synthesis. Collectively, the failure of winter anovulatory mares to respond to continuous infusion of native GnRH could not be attributed to differences in the magnitude or pattern of LH and FSH secretion compared to responsive mares. A GnRH challenge administered to ovarian-unresponsive GnRH + High E2 mares and Control mares revealed an inability of the former to release additional gonadotropin whereas controls, not exposed previously to continuous GnRH treatment, did exhibit a measurable increase in both LH and FSH. The latter is indicative of the small releasable pools known to exist in winter anovulatory mares. The failure to release additional gonadotropins in the unresponsive GnRH + High E2 mares suggests a maximal stimulation of pituitary gonadotropes. In the equine scenario, this is manifested by a chronically elevated but subdued LH baseline capable of stimulating follicle growth but unable to respond to further stimulation because of receptor down-regulation. In the case of FSH, maximal stimulation was characterized by an acute, short-lived peak in

FSH release followed by a return to baseline, also indicative of receptor down-regulation or a small releasable pool of FSH within the pituitary. Taken together, these responses suggest that the chronic infusion of GnRH favors the synthesis and secretion of LH while differentially down-regulating FSH synthesis and secretion at a much faster pace. It should be noted that a follicular wave was stimulated in all mares treated with GnRH regardless of E2 treatment. However, follicular diameter in unresponsive GnRH-treated mares did not exceed 25-mm and regressed, indicating that conditions necessary for follicular deviation were not present. Further investigations into this phenomenon are warranted.

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APPENDIX

Methodology

Equine progesterone (P4) RIA

1. Iodinated Product: [^{125}I]-Progesterone (from MP Biomedical CT kit #07-270102 and #07-270105)

2. Antibody: Coated tube anti-progesterone (MP Biomedical, CT kit #07-270102 and #07-270105)

3. Standards (stds): kit provided standards (0.15 – 20.0 ng/ml)

    a. Two additional standards were made: 2.0 ng/ml and 10.0 ng/ml by serially diluting the kit-provided 80.0 ng/ml standard with the excess 0.0 ng/ml kit provided standard.

4. References (refs): Progesterone added to winter anovulatory equine plasma (1.0, 5.0, and 10.0 ng/ml)

5. Procedure:

    Day 1:

a) Pipette 100 µL of each STANDARD, SAMPLE and CONTROL into its respective coated tube.

b) When all samples have been pipetted, add 1.0 mL of PROGESTERONE-^{125} to all tubes and vortex briefly.

c) Incubate all tubes: Incubate at 4° for 16-24 hours

Day 2:

d) Aspirate or decant the contents of the tubes in the same order as pipetted. (If decanting, blot the tube on absorbent paper before turning upright.)

Decanting procedure: Have a basin for decanted liquid as well as a disposable, absorbent blotting material ready. Using a foam 12x75mm tube holder (holds 100 tubes/holder), place all tubes besides TC into the holder. In a quick fluid motion, flip the holder with the tubes over the basin and allow liquid to empty from tubes. Immediately blot the tubes on the absorbent material and then invert them upright.

e) Count the tubes in a gamma counter

Equine luteinizing hormone (LH) RIA

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

2. Antibody: Anti-equine LH (AFP-240580). Dilution 1:120,000

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 follicle-stimulating hormone (FSH) RIA

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 (2.0 and 8.0 ng/ml)

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.

Equine estradiol-17β (E2) RIA

1. Iodinated Product: [^{125}I]-estradiol-17β (conjugated to 3-position, MP Biomedical product #07-138228)

2. Antibody: Anti-estradiol-17β (conjugated to 3-position, MP Biomedical #07-138216). Diluted to 1:150,000

3. Extraction solvent: Methyl-tert-butyl-ether (MTBE, HPLC grade, Fisher Scientific)

4. Standards (stds): Constructed from 50.0 ng/ml estradiol-17β in methanol (MeOH). Various volumes (described below) pipetted into 12 x 75 mm borosilicate glass tubes dried down and rehydrated (1.25 – 150.0 pg/ml)

5. References (refs): Estradiol-17β (E2) added to pooled equine plasma. A calculated amount of 5,000 pg/ml E2 in MeOH was dried down and rehydrated with equine plasma to constitute a 100 pg/ml reference (high ref). The high ref was serially diluted with equine plasma to create 2.5, 5.0, and 50.0 pg/ml references. A baseline (no added mass) ref was included in each assay.

6. Extraction Procedure

I. Tracer Preparation – *Prepare prior to recovery estimates* (Using "bulk TE tracer")

1. Dilute bulk stock tracer as per MP Biomedical instructions to yield a 1:300 stock solution.
2. In triplicate, count 100 µL of 1:300 stock.
3. Calculate the needed amount of tracer for the assay (100 µL/tube of 5,000 cpm/100 µL tracer).
4. Using only what is needed for the current assay, dilute a portion of the 1:300 stock tracer to obtain 5,000 cpm/100 µL using the equation below.

(cpm/.1 ml in 1:300)x = (5,000 cpm/.1 ml)(ml of tracer needed for assay)

II. Recovery Estimates – *Prepare at least 30 min prior to extraction*

1. Pipette 1500 cpm ^{125}I-estradiol (diluted prior to assay) into charcoal-stripped (or baseline) plasma/serum of same volume as unknowns/references.
    a. Keep volume of tracer per ml low (25-100 µl)
    b. Mix and allow to equilibrate at 4°C for *at least* 30 minutes before extracting
    c. Make 3 total count recoveries (TCR) using 12x75mm culture tubes using same volume of tracer (25-100 µl) used for recovery estimates
        i. These **will not** undergo the extraction process – keep with TC's

III. Extraction Set-Up

1. Pipette 0.3 ml of unknown and reference samples into 16x100 mm Pyrex tubes in duplicate.
2. Add 4 ml of methyl-tert-butyl ether (MtBE, HPLC grade) to each reference, unknown, blank and recovery-estimate tube.
3. Vortex for 1 minute using multi-tube vortexer or for 30-40 minutes using an end-to-end shaker.

IV. Separating/harvesting solvent from extracted serum/plasma and blank tubes

1. Following vortexing allow tubes to sit upright at room temperature for 15 minutes to allow phases to separate.
2. Place tubes in ultra-low freezer (-80°C) for 20 minutes or as long as required for the plasma/serum to freeze solid.
    a. Alternatively, a dry-ice/methanol bath or liquid nitrogen can be employed to accomplish this quickly.
3. Remove frozen samples, place on ice, and quickly decant the supernatant into 12x75mm borosilicate assay tubes to avoid thawing of plasma/serum.
    a. ***NOTE:*** It is recommended to only extract one rack (~72 tubes) at one time to avoid thawing of serum during this procedure (MMO, June 2017)

V. Drying extracts

1. Dry references, unknowns and blanks to complete dryness under air in the hood. Samples can be heated to 37°C if desired using a heating block. ***Do not*** over-dry

a. KLCT403 – Compressed N_2 can be fed into the drying apparatus under the fume hood in KLCT401.

2. Extracts from ***recovery estimates*** should be dried partially to minimize the chance of spillage (0.5 – 1 ml) but need not be dried completely. Rehydrate with 100 µl 1% BSA.
    a. Set aside with TCR and TC after this step

VI. Rehyrdation of extracts

1. Rehydrate ***references*** and ***unknowns*** with 37°C assay buffer (1% BSA) to bring to volume of 100 µl and vortex. Cover and place in refrigerator until ready to proceed with assay.

VII. Standard Curve Tubes

1. Using 12x75mm borosilicate glass tubes, pipette 4 ml MTBE into the standard curve tubes (#10-39). Do not expose NSB tubes to MTBE.
2. Using the drying rack, dry down standard tubes to complete dryness.
3. ***Once all other extracted tubes are dried and rehydrated***, pipette the following volumes of 50 pg/ml E2 in methanol into the respective standard tubes:

    i. 1.25 pg/ml 5 µl
    ii. 2.50 pg/ml 10 µl
    iii. 5.00 pg/ml 20 µl

iv.	12.5 pg/ml	50 µl
v.	25.0 pg/ml	100 µl
vi.	37.5 pg/ml	150 µl
vii.	50.0 pg/ml	200 µl
viii.	100.0 pg/ml	400 µl
ix.	150.0 pg/ml	600 µl

4. Once completely dried, rehydrate with 100 µl warm (37 °C) 1% BSA.

7. RIA Procedure

I. Assay Set-Up

A. Label assay sheets and number the tubes (12x75 mm borosilicate glass tubes and 12x75mm plastic recipient tubes) and counting vials (plastic vials; 7 ml) as follows):

Tubes:	Tube Numbers:
a) 5 TC tubes	1-5
b) 4 NSB	6-9
c) 3, 0 STD	10-12
d) Triplicate for each standard	13-39
e) 3 Baseline	40-42

e) 3 Lowest ref 43-45

f) 3 Low ref 46-48

g) 3 Mid ref 49-51

h) 3 High ref 52-54

i) 3 Blanks 55-57

j) 3 Recoveries 58-60

k) 3 TCR 61-63

l) Triplicate unknowns

II. Day 1

A. Pipette the following volumes of 50 pg/ml E2 in methanol for each respective standard tube:

1) 1.25 pg/ml 5 µl
2) 2.50 pg/ml 10 µl
3) 5.00 pg/ml 20 µl
4) 12.5 pg/ml 50 µl
5) 25.0 pg/ml 100 µl
6) 37.5 pg/ml 150 µl
7) 50.0 pg/ml 200 µl
8) 100.0 pg/ml 400 µl
9) 150.0 pg/ml 600 µl

As with extractions, dry-down the tubes completely using the drying rack under compressed nitrogen. Do not allow the tubes to over-dry.

Rehydrate each tube with 100 µl warm (37°C) 1% BSA.

B. Pipette the following into each tube:

1)	NSB	200 µl 0.1% PBS-Gel
2)	0 Standard	100 µl 1% BSA
3)	STD's	Respective E2-methanol volume dried-down
		Rehyrdated with 100 µl 1% BSA

C. Once the tubes containing plasma/serum extracts are dried, bring to assay volume (rehydrate) of 100 µl using warm 1% BSA and vortex briefly to re-dissolve estradiol dried to the walls of the tubes.

D. Pipette 100 µl E_2 antiserum (1^{st} antibody) into all tubes **except NSB's, recoveries, and TC recoveries (TCR).** Working dilution of antiserum is to be determined in advance of the assay.

E. Incubate for 1 hr ***on ice*** in the refrigerator at 4°C before adding tracer

F. Pipette 100 µl ^{125}I-E_2 (5000-6000 cmp/100 µl) into all tubes including TC vials.

   1) Pre-dilute the TE tracer prior to assay

G. Vortex tubes briefly, cover with aluminum foil and incubate for approx.. 20 hours at 4°C.

III. Day 2

A. Add 1.0 ml of ice cold **Charcoal-Dextran suspension** (kept in an ice bath and constantly stirring) to ice-cold tubes (in an ice bath).

NOTE: Add charcoal only to the number of tubes that the centrifuge carriers will accommodate for each run. **DO NOT ADD CHARCOAL TO TCR OR RECOVERIES!**

B. Immediately vortex all tubes.

C. Incubate tubes for 50 minutes at 4°C at 3600 rpm in the Beckman J-6 M/E (JS-4.2 Rotor).

D. Decant supernatant from each glass tube into corresponding plastic 12x75mm tubes. Try not to spill around the edges of recipient tube. Wipe tubes with Kim Wipe before loading into gamma counter to avoid contamination.

E. Count assay using a programmed estradiol protocol on gamma counter.