REPRODUCTIVE NEUROENDOCRINE FUNCTION IN THE MARE AS REFLECTED IN THE INTERCAVERNOUS SINUS DURING OVULATORY, ANOVULATORY, AND TRANSITIONAL SEASONS

A Thesis

by

DEE A. COOPER

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

MASTER OF SCIENCE

May 2006

Major Subject: Physiology of Reproduction

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Approved by:

Chair of Committee,	Gary L. Williams
Committee Members,	Paul G. Harms
	Katrin Hinrichs
Head of Department,	Gary Acuff

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ABSTRACT

Reproductive Neuroendocrine Function in the Mare as Reflected in the Intercavernous Sinus during Ovulatory, Anovulatory, and Transitional Seasons.

(May 2006)

Dee A. Cooper,

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Chair of Advisory Committee: Dr. Gary L. Williams

We hypothesized that marked reductions in secretion of luteinizing hormone (LH) during transitional and anovulatory periods can be accounted for by similar reductions in hypothalamic gonadotropin-releasing hormone (GnRH) secretion. Catheters were inserted surgically into the intercavernous sinus (ICS) of seven non-pregnant mares via the superficial facial vein during the ovulatory season (August 12-23), fall transition (November 15-30), the anovulatory season (January 19 - February 1) and spring transition (March 24 - May 12). Catheter placement was confirmed and standardized in each mare by lateral radiography. Ovarian status was monitored throughout the study by transrectal ultrasonography and serum concentrations of progesterone. During the breeding season, ICS blood samples were collected at 5-min intervals for 8 h when the dominant follicle reached approximately 35 mm and estrous behavior was observed. All mares ovulated within 5 d after sampling, except one mare who ovulated < 24 h before sampling. During the fall, mares were anovulatory (n = 5) or had a final ovulation within 5 d following intensive sampling (n = 2). Winter anovulation

sampling was performed when all mares were anovulatory. During spring transition, each mare was sampled just before the second ovulation of the season. Similar to the ovulatory season, mares were sampled when the dominant, preovulatory follicle reached approximately 35 mm and estrous behavior was observed. Mean concentrations of LH were markedly higher ($P < 0.01$) during the breeding season than during all other seasons. Lower mean concentrations of LH in the fall transition, winter anovulation and spring transition sampling periods occurred coincident with a similar reduction ($P < 0.01$) in amplitude of LH pulses. Unexpectedly, neither the frequency (pulse/8 h) of LH pulses, frequency and amplitude of GnRH pulses, nor mean concentrations of GnRH differed among seasons. In addition, there were no differences observed due to season in mean concentrations of FSH or amplitude of FSH pulses. However, a small but significant ($P < 0.05$) reduction in the frequency of FSH pulses was observed during fall transition compared to all other seasons. In summary, contrary to accepted dogma, these results indicate that the photoperiodic initiation of seasonal anovulation in the mare is mediated at the level of the anterior pituitary, and appears to occur through a dampening of gonadotroph responsiveness to an unchanging pattern and magnitude of GnRH secretion.

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CHAPTER I

INTRODUCTION

Mares are seasonal long-day breeders with a loosely defined natural breeding season that extends from April to October in the Northern Hemisphere (1). A majority of breed and racing associations have imposed an arbitrary birth date of January 1 on foals born in the Northern Hemisphere (1). This universal birth date creates problems for horse breeders because of the asynchrony between the natural and the operational breeding season, and the need to have foals born as early in the calendar year as possible in order to have two-year olds mature enough for competition. In order for a foal to be born at the optimal time, as soon after January 1 as possible, the mare must conceive in February when she is typically anovulatory. Therefore, much research has focused on hastening the onset of the first spring ovulation. One method that has been investigated is the administration of gonadotropin-releasing hormone (GnRH) or its analogues using various dosages and regimens (2-13). These treatment regimens have been somewhat successful, but not in a practical, applicable manner that has resulted in their commercialization. Artificial lighting programs have been the most successful and widely used approaches to control reproductive seasonality in mares (14-21). Although these programs are generally successful, there are limitations to their practical use,

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This thesis follows the style of Endocrinology.

including costs associated with the time, labor and facilities needed to implement them (1).

In order to gain a better understanding of reproductive seasonality in the mare and to develop better strategies for managing it in a practical manner, more information is needed about the specific mechanisms within the brain that regulate hypothalamic GnRH and pituitary gonadotropin secretion in response to changing photoperiod. Gonadotropin-releasing hormone, released from the hypothalamus, controls the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary. Studies involving the direct measurement of GnRH in animals, including the mare, are not abundant. However, due to the unique vascular architecture of the equine cranium, GnRH secretion can be measured from blood that drains into the intercavernous sinus (ICS), which can be reached through cannulation of the superficial facial vein (22).

Previous work in our laboratory (23) has shown low-dose, native GnRH, delivered sc via a continuous osmotic pump to lactational and idiopathic anovulatory mares, to be an effective treatment to induce development of an ovulatory follicle and ovulation. Within 6 wk, 80 to 85% of mares treated sc with GnRH at 2.5 to 5 μg/h had ovulated and been bred compared to 12% in untreated control mares, indicating the effectiveness of this approach for inducing ovulation in anovulatory mares during the operational breeding season. However, continuous treatment with GnRH is incapable of preventing the onset of seasonal anovulation. Morton et al. (24) applied continuous GnRH treatment to cyclic mares beginning in the early fall, and continuing throughout the winter and

early spring. All mares became anovulatory by December 1, and mean concentrations of LH and FSH in peripheral blood were not affected by GnRH treatment. These results suggest that doses of GnRH effective during spring transition and the breeding season are not effective for maintaining ovulatory cycles during the anovulatory season.

To understand these relationships more fully and to design treatments that might allow effective management of seasonal anovulation, it is necessary to increase our understanding of the seasonal regulation of endogenous GnRH and LH secretion, including the role of pineal melatonin. Current dogma suggests that the lack of an adequate GnRH signal to the anterior pituitary is the cause of seasonal anovulation (1). This is based on indirect but limited data in the mare and rather expansive information from other species such as the seasonal breeding ewe. Therefore, one would expect the secretion of pulsatile GnRH to be highest during the ovulatory compared to the anovulatory season, and to decrease markedly during transition into and out of the ovulatory season, coincident with the well-documented suppression of LH secretion. However, this has not been clearly demonstrated in the mare, and the overt refractoriness of the equine anterior pituitary to exogenous GnRH during the fall and winter seasons in most mares suggests that there are other mechanisms regulating this phenomenon. Moreover, even if the hypothesis that GnRH is limiting during seasonal anovulation is correct, more precise GnRH treatment regimens could potentially be developed if the basic reproductive neuroendocrinology of seasonal anovulation were better understood.

The unique manner in which pituitary venous blood drains into the intercavernous sinus of the horse makes cannulation of this site the first choice for

measuring the acute release of GnRH in the equine. This is the only species known in which the arrangement of the vasculature allows for cannulation of the superficial facial vein in an easily-accessible location. Although hypothalamic *content* of GnRH has been well characterized, the *secretory* pattern of GnRH in a significant number of mares throughout the ovulatory and anovulatory seasons has not been clearly established. Moreover, limitations in our knowledge of pulsatile secretion of LH in the mare also exist, as the detection and characterization of distinct pulses in peripheral blood is not possible except during the luteal phase when pulses are quite large. In addition, concentrations of FSH have been shown to have little seasonal variation (1) but will ultimately complete the overall picture obtained. Therefore, the purpose of this study was to acutely monitor the pulsatile pattern of GnRH, LH and FSH secretion in venous effluent directly below the lower brain and pituitary of the mare during distinct periods: the ovulatory and anovulatory season, and during spring and fall transitions into and out of the ovulatory season.

CHAPTER II

LITERATURE REVIEW

Hormonal and Behavioral Characteristics of Reproductive Seasonality

Fall Transition

Reports characterizing physiological and endocrine events associated with transition from the ovulatory to the anovulatory season in the mare are limited. In general, the ovulatory season terminates in October or November and is characterized by failure to develop a follicle or failure to ovulate a developing follicle (25). Snyder et al. (26) investigated follicular and gonadotropic hormone differences between the period following the next to last, or penultimate ovulation and the period after the final ovulation of the ovulatory season in 14 pony mares. There was no difference in plasma concentrations of FSH, numbers of large follicles, or estrus behavior between periods. However, concentrations of LH were lower following the final ovulation than the penultimate ovulation. This group also reported that several mares appeared to continue some ovarian activity following the last ovulation, exhibiting follicular growth and estrous behavior, but failed to ovulate.

Anovulatory Season

Anatomically and histologically, the anovulatory season is associated with an absence of large follicles on the ovary (27, 28), small, firm ovaries, minimal folding of cervix and endometrium, and a toneless uterus that is difficult to palpate (28). Turner et

al. (29) reported on follicular and gonadotropic hormone changes throughout the year in pony mares. The monthly means for the diameter of the largest follicle, overall number of follicles, and mean concentrations of LH showed a seasonal pattern, with mean values for April to October being higher than for the anovulatory months. Other work has supported these findings, with mean concentrations of LH minimal during the anovulatory period, comparable to the low concentrations typical of mid-diestrus during the estrous cycle (30). However, monthly mean concentrations of FSH do not appear to differ between seasons of the year (29).

Spring Transition

The latter portion of the anovulatory season is associated with an increase in the number of 15-25 mm follicles (29, 31). This is associated with a high mitotic index, a measure of the extent of cell division, for preantral follicles at this time (27). Eventually, the number of small follicles decreases and the number of large follicles and the diameter of the largest follicle increases rapidly between 8 and 1 d before ovulation (29, 31). During this time period, many mares have been reported to exhibit follicular waves, with one or two follicles reaching preovulatory dimensions, but regressing before development of a similar large follicle that ovulates (32, 33). In addition, before emergence of follicular waves, which are identified by the presence of a dominant follicle, follicular activity has been described as erratic with no follicles reaching 35 mm (33). Morphological differences between transitional and preovulatory follicles have been investigated (34). Follicular angiogenesis is necessary for survival of preovulatory

follicles, and transitional follicles have been found to have less vascularization and less proliferative activity than preovulatory follicles (34).

For many mares, the ovulatory season is preceded by a period of prolonged estrous behavior, which includes days when mares are unresponsive to a stallion (31, 32). However, serum concentrations of LH do not increase until just before the first ovulation of the season (31). Turner et al. (29) have reported that monthly mean FSH concentrations do not differ over a 12-mo period, but other groups have shown that FSH concentrations and pulse amplitudes decrease during spring transition (31, 35). In general, duration of the first estrous cycle of the season is comparable to normal estrous cycles during the middle of the breeding season (32). However, this is in contrast to seasonal breeding ewes, which typically exhibit one or more short luteal phases at the onset of the breeding season before establishing normal cyclic activity (36).

Neuroendocrine Basis of Seasonality

Role of Melatonin in Seasonal Breeding

Understanding the physiological mechanisms controlling seasonal reproduction in the mare has been a long-term scientific goal, with many questions remaining unanswered. Melatonin, a hormone produced and secreted by the pineal gland, appears to play a central role in seasonal reproduction but its actions in this regard are not well understood. Melatonin secretion is known to be controlled by photoperiod, with the greatest secretory activity occurring during dark hours (1) and the onset signaled by the onset of dusk (37). When melatonin secretion increases during periods of decreasing day length, the mare typically transitions into the anovulatory state. Melatonin treatment has

been shown to decrease GnRH content of the hypothalamus (38), and melatonin implants during the summer produced a brain tissue content of GnRH similar to normal winter values (39). However, Fitzgerald and McManus (40) found that the administration of melatonin to mares failed to significantly alter the onset of the anovulatory period.

Sheep are also seasonal breeders, with the timing of their breeding season being controlled by photoperiod and melatonin. However, unlike mares, sheep are short day breeders (41), and melatonin therefore has a stimulatory effect on cyclic activity. Exogenous melatonin treatment has been shown to advance the onset of the breeding season in ewes (42). However, this successful advancement of breeding activity was not reflected in peripheral LH and FSH concentrations at this stage (42). Barrell et al. (43) have demonstrated that the mere presence of melatonin is not adequate to entrain the circannual reproductive rhythm in the ewe. Rather, the characteristic of the melatonin pattern is crucial in order to elicit a response.

The mechanism by which melatonin signals seasonality in sheep is not well understood, but recent evidence has implicated the premammillary hypothalamic area (PMH) as a key site for the influence of melatonin on reproductive seasonality. Sliwowska et al. (44) have characterized the distribution of neuronal divisions in the PMH of sheep. These findings should help guide future work in this species and in other seasonal breeders for determining the precise role of melatonin in these processes.

Role of the Thyroid Gland in Seasonal Breeding

The thyroid gland has been found to be an important factor controlling seasonal

breeding in sheep. Several groups (45, 46) have shown the thyroid to be essential for ewes to transition into anestrus. Thyroidectomized ewes had a frequency of pulses of LH similar to thyroid-intact ewes during the breeding season. However, at the end of the breeding season, circulating LH declined to basal concentrations in thyroid-intact ewes, but values remained at breeding season levels in thyroidectomized ewes (45). While it is quite clear that the thyroid is required for initiation of anestrus in ewes, Thrun et al. (47) have demonstrated that thyroid hormones are not obligatory to maintain anestrus once it has been established. In addition, thyroid hormones do not control the onset of a subsequent breeding season in the ewe (47).

Through administration of thyroxine to thyroidectomized ewes, evidence has developed suggesting that thyroid hormones may act at the brain to inhibit the secretion of LH, resulting in anestrus (48). Thyroxine infused centrally was effective in causing this effect, but not when infused peripherally. Thyroxine replacement does cause anestrus in thyroidectomized ewes (46, 48), but the presence of thyroid hormones to cause anestrus is necessary only late in the breeding season and responsiveness to thyroxine is lost gradually during the mid to late anestrus season (46, 49). A more applied study investigated the use of propylthiouracil (PTU) to induce thyroid suppression in order to extend the breeding season in ewes (50). At the conclusion of the 35-d treatment period, 25, 60 and 100% of ewes receiving 0, 20 or 40 mg of PTU/kg BW continued to exhibit normal estrous cycles (50).

In contrast to the ewe, the role of the thyroid in seasonal breeding in the mare has not been thoroughly investigated. It has been shown that thyroid hormones reach their

highest concentrations in the circulation during the winter, when mares are typically anovulatory (51). Further research into the action of the thyroid in mares could prove useful in gaining a better understanding of seasonal breeding in this species.

GnRH Receptors

The hypothalamic-hypophyseal neuroendocrine axis serves as the master control center of reproduction in mammals. Releasing hormones, including GnRH, from the hypothalamus must reach the adenohypophysis via a capillary network (hypophyseal portal vessels) in the infindibulum. Receptors for GnRH are located on adenohyphyseal gonadotrophs and are necessary for normal synthesis and secretion of LH and FSH to occur. Gonadotrophs are either monohormonal (produce either LH or FSH) or bihormonal (produce both LH and FSH) (52). The concentration of gonadotrophs in the anterior pituitary does not appear to differ between estrous cycling and anestrus mares (52, 53). Similarly, mares are unique in the fact that concentration and content of GnRH receptors on the anterior pituitary are not affected by season (54, 55).

The equine pituitary also has the unique ability to resist desensitization to GnRH, which commonly occurs in other species when the pituitary is exposed to supraphysiological or chronically-elevated concentrations of GnRH (56, 57). Porter et al. (58) compared equine and ovine LH secretory responses to continuous and pulsatile treatment with GnRH. Mares exhibited elevated secretion of LH with both treatment regimens. Secretory patterns of LH exhibited by ewes treated with pulsatile GnRH did not differ from ovariectomized controls. However, continuous treatment of ewes with GnRH eventually caused reduced secretion of LH due to GnRH receptor down-

regulation. Porter and Sharp (59) have also shown that the equine pituitary provides GnRH receptors to the cell surface even in the presence of continuous GnRH exposure. The rate of endocytosis of the equine GnRH receptor appears to be much slower than that of other species (59). Interestingly, the equine receptor gene is very similar to other species, but slight differences observed in the amino acid sequence may serve as a basis for its ability to resist down-regulation.

Hypothalamic and Pituitary Content of GnRH, LH and FSH

Hypothalamic content of GnRH and anterior pituitary contents of LH and FSH in the mare during different physiological states have been reported (52, 54, 55, 60, 61). Silvia et al. (60) found the content of GnRH in the median eminence of seasonally anovulatory mares to be lowest 1 wk prior to and 12 wk following the winter solstice. The latter decline in hypothalamic concentration of GnRH may have been observed because of a renewed release of GnRH occurring as a result of the development of a state of photorefractoriness. The latter term represents a state that occurs when there is a spontaneous reversion in physiology to that of the opposite photoperiod (62). Refractoriness to the previously decreased photoperiodic environment following the winter solstice is a phenomenon also seen in other seasonally breeding species (63). Commencement of the breeding season in the ewe is not actively driven by decreasing day length, but by refractoriness to prevailing long days.

Other groups have observed no effect of season on total GnRH content in the hypothalamus or concentration of receptors for GnRH in the pituitary of the mare (55, 61). Similar to hypothalamic GnRH, the concentration of FSH in the anterior pituitary is

not affected by season (55). However, anterior pituitary concentrations of LH have been reported to vary markedly depending on the time of year (55). This suggests that the synthesis of LH is the primary limiting factor driving the seasonal anovulatory state in the mare. However, collective reports on content of GnRH in the hypothalamus and LH and FSH in the anterior pituitary have been inconsistent and limited, and do not provide an adequate or clear assessment of secretory patterns associated with changing seasons.

GnRH Secretion

Determining concentrations of hypothalamic GnRH and pituitary concentrations of LH and FSH does not provide a measure of secretion of these hormones. Therefore, it is impossible to make relevant, physiological inferences concerning the secretory dynamics of GnRH, LH, or FSH in mares based on published literature. In addition, due to rapid degradation, GnRH cannot be measured in peripheral blood plasma or serum of mammals. To overcome this limitation, Sharp and Grubaugh (64) utilized the technique of push-pull perfusion, which involves inserting a guide cannula into the medial basal hypothalamus and perfusing a carrier medium through the tissue to estimate hypothalamic GnRH secretion in conscious horses. Secretory rate of GnRH was found to increase as mares progressed from winter anestrus to estrus in the breeding season. However, monitoring GnRH in brain perfusates at 10-min intervals demonstrated an irregularly episodic release pattern without any apparent regular pulsatility observed. Moreover, measurement of LH in concurrent jugular samples failed to demonstrate obligatory temporal peak agreement (64). Therefore, the data reported from these

experiments contribute very little to our understanding of reproductive neuroendocrine mechanisms regulating gonadotropin secretion in the mare.

In 1984, Irvine and Hunn (65) published a preliminary report describing a new and unique procedure for collecting pituitary venous effluent in the horse. This technique involves cannulation of the intercavernous sinus (ICS) through the superficial facial vein, with the entry site located proximal to the mandible (65). Further work (22) described the ICS cannulation technique in much more detail. Cannulation of the ICS is minimally-invasive and can be performed using only a local anesthetic. Sampling from an ICS cannula disturbs the animal no more than collection of a jugular blood sample, and allows the monitoring of secretory hormone activity under completely physiological conditions. Use of this method for measurement of pulsatile GnRH, LH and FSH secretion is possible only in equids due to the unique venous drainage of the pituitary and the arrangement of the equine cranial vasculature (22). Blood collected with this technique for measuring concentrations of GnRH first passes through the pituitary. Alexander and Irvine (22) examined this possible disadvantage and concluded that the fraction of GnRH removed by the equine pituitary is minimal.

Irvine and Alexander (22) also evaluated and described some potential problems with the ICS cannulation procedure. During the actual cannulation process, there can be difficulty maneuvering the cannula into the ventral branch of the ophthalmic vein where it narrows to enter the orbital foramen and becomes the cavernous sinus. At times, it proved difficult to cause the cannula to progress toward the cranium into the deep facial vein. As a result, it turned downwards into one of the superficial nasal veins. Because of

these potential obstacles, radiographic confirmation of the cannula position following placement proved to be useful. Other concerns of these investigators included the effects of chewing, changes in head position, or vascular reactions to the cannula itself on blood flow and the resulting evaluation of hormone secretion. Results of their experiments indicated no consistent effects of any of these factors on concentrations of hormones in ICS effluent.

Since the discovery and publication of the ICS cannulation technique, only a few attempts have been made to quantitate the pulsatile secretion of GnRH, LH and FSH during various periods of the ovulatory and anovulatory season in mares. Silvia et al. (66) examined the release of the gonadotropins in pituitary venous blood during the early follicular phase, immediately following luteolysis. Peaks of LH and FSH were observed to occur at an approximate hourly rate within 36 h following induced luteal regression, when mean concentrations of progesterone were less than 1 ng/ml (66). Also, pulses of LH and FSH and mean baselines of these hormones measured in pituitary venous blood have been reported to exhibit up to 100 fold greater magnitudes than in corresponding jugular blood samples (66, 67).

During the preovulatory, follicular phase increase in LH of the mare, as measured using 30-sec sampling of pituitary venous blood, GnRH was observed to be secreted in frequent (4.3/h) brief (5-min) peaks (68). Peaks of LH and FSH occurred at a frequency of 4.6 and 1.9 peaks/h, respectively (68). Similarly, peaks of LH and FSH have been shown to occur concurrently in 5-min ICS samples, with pulse frequency varying from 0.45 pulses/h on d 4 to 6 before ovulation to 1.87 pulses/h on the day of

ovulation (67). During the follicular phase, LH and FSH pulses were secreted in unison 83% of the time, and 98% of LH pulses were associated with a GnRH pulse (67, 68) in ICS samples.

The ovulatory surge of the mare is unique because peripheral peak concentrations of LH do not typically occur until 1-2 d post ovulation (1). Greaves et al. (69) have reported the release of GnRH and LH during this period following ovulation. They found concentrations of GnRH in samples taken from the ICS to decrease by d 8 post-ovulation in both intact and ovariectomized mares, and circulating concentrations of LH decreased by 3 d post-ovulation as serum concentrations of progesterone rose. Likewise, Irvine and Alexander (70) demonstrated that the persistence of the ovulatory surge of LH in mares is due to continued secretion of LH and FSH accompanied by GnRH, which is eventually decreased as negative feedback by progesterone increases from the newly formed corpus luteum.

Concentrations of plasma LH and FSH from jugular blood samples collected during the luteal phase have demonstrated pulse frequencies of 3 and 1 peaks per 24 h, respectively (71). Irvine and Alexander (72) reported similar results in pituitary venous blood samples taken during the mid luteal phase, about 6-12 d after the end of estrus. Episodes of GnRH, LH and FSH secretion were predominantly concurrent and had large amplitudes with prolonged (30-55 min) episodes that were the result of 3 to 6 individual peaks of declining magnitude (72).

Jochle et al. (73) reported on GnRH, LH and FSH secretion patterns in pituitary venous blood of ICS samples collected during the spring transition into the breeding

season. Peaks of LH were observed to be 8-104 times greater and FSH peaks 3-5 times greater in pituitary venous blood than in the corresponding jugular samples. In addition, during the 24-h sampling period, pulses of GnRH, LH, and FSH were very infrequent and did not occur at all in two mares in the study. The reported findings of this group are the only publications of ICS sampling in mares during the transition period.

Studies describing the secretion of GnRH in a significant number of mares throughout the ovulatory and anovulatory seasons have not been reported. However, seasonal changes of GnRH secretion have been reported in the ewe (74). Pituitary portal blood was measured for concentrations of GnRH for 5 ewes during the follicular phase of the estrous cycle during the breeding season and 5 ewes in the anovulatory state. During the breeding season, ewes exhibited 8 pulses of GnRH/6 h, compared to < 1 pulse/6h during the anovulatory season (74). This evidence clearly demonstrated that the seasonal anovulatory state in ewes is a result of the absence of high-frequency pulses of GnRH.

Control of Seasonal Breeding in Mares

Artificial Lighting

Artificial lighting programs have been shown to alter ovarian cyclicity in mares. Some of the earliest studies in this area were conducted on Thoroughbred and Standardbred farms in Kentucky. In one study, light treatment began on November 27, with 12 h light/d and increasing to 19 h light/d by May 6. Thirty-six % of the lighted group and none of the unlighted group became pregnant by March 31 (14). Cleaver et al. (15) found that mares exposed to constant light for 28 d in October in the Northern

Hemisphere had significantly higher concentrations of LH in daily blood samples and during frequent sampling periods than did control mares exposed to 12 h of light and 12 h of dark. Also, GnRH content in the hypothalamus was 1.6 pg/mg protein in the treatment group compared with 0.3 pg/mg protein in controls (15). Through a quite extensive study on 100 Standardbred mares in the U.S., Cooper and Wert (16) found 16 h of light and 8 h of darkness to be an effective method for inducing breeding activity in mares during winter. After 5 yr, 50% of foaling occurred during November to January as compared to 3.8% at the onset of the program. Other groups have also demonstrated that an artificial photoperiod of 16 h of light and 8 h of darkness, beginning in December, produces the most desirable results and can result in normal estrous cycles within two months (17, 18, 19). Oxender et al. (17) reported that mares in an indoor light-treated group (16 h light, 8 h dark) ovulated at least 74 d sooner than outdoor control mares. In addition, indoor light-treated mares averaged 4.2 estrous cycles prior to the end of April, and winter hair was shed earlier than controls. Kooistra and Ginther (19) also showed that 16 h of light and 8 h of dark was an effective method for hastening the onset of the ovulatory season. The interval to first ovulation was significantly shorter for mares with active ovaries (follicles > 15 mm) than for mares with inactive ovaries at the onset of light treatments. Kennedy et al. (20) have made several recommendations for establishing a successful lighting program. Their recommendations include the use of one 200 watt incandescent bulb to provide 2 ft candles of light intensity per stall, the use of automatic timers, and increasing the amount of light by 30 min/wk until mares are receiving 16 h of light (artificial plus natural) per 24 h. In conclusion, artificial lighting

programs can be very successful; however, as stated previously, they may not be practical for all producers.

Treatment of Mares with Exogenous GnRH

In addition to the use of artificial lighting, other methods for accelerating the onset of the first ovulation of the year and to induce ovulation during the breeding season in mares involves the administration of GnRH. Previous studies of seasonally anestrous mares have shown that administration of native GnRH in a pulsatile fashion, with doses ranging from 2 to 250 μg/h, has resulted in ovulation within 12 d following the start of treatment (3, 9). Hyland and Jeffcott (8) reported that sc infusion of GnRH via osmotic minipumps at a rate of 40-60 μg/h for 28 d resulted in first ovulation approximately 5 wk earlier than control mares.

A slow-release implant containing the GnRH analog, deslorelin, has been found to be an effective, consistent method of inducing ovulation in cyclic mares during the breeding season (13) and is currently marketed as Ovuplant (Ft. Dodge Animal Health, Overland Park, KS). Likewise, Williams et al. (23) have shown low-dose, native GnRH, delivered sc via a continuous osmotic pump to lactational and idiopathic anovulatory mares, to be an effective treatment to induce development of an ovulatory follicle and ovulation. Within 6 wk, 80 to 85% of mares treated sc with GnRH at 2.5 to 5 μg/h had ovulated and been bred compared to 12% in untreated controls, indicating the effectiveness of this approach for inducing ovulation in anovulatory mares during the operational breeding season. However, Mumford et al. (6) reported that use of a GnRH analog in implant form, administered at varying doses, resulted in only 15 of 100

transitional mares ovulating within 30 days of treatment. Similarly, Fitzgerald et al. (10) found that constant administration of the GnRH agonist, goserelin acetate, via a biodegradable depot, failed to provide an adequate level of reproducibility or effectiveness for commercial use in anovulatory mares. In addition, Morton et al. (24) showed that continuous treatment with GnRH is incapable of preventing the onset of seasonal anovulation by applying continuous treatment to cyclic mares beginning in the early fall, and continuing through the winter and early spring. All mares became anovulatory by December 1, and mean concentrations of LH and FSH were not affected by GnRH treatment.

One previous study of cycling mares has shown that administration of GnRH in a pulsatile manner beginning on day 16 of the estrous cycle can advance ovulation (12). Similarly, Harrison et al. (4) reported ovulation in 7/15 transitional mares following the pulsatile infusion of the GnRH agonist, buserelin, for 28 d compared to 0/15 ovulations in untreated control mares. Therefore, while GnRH administration can be effective in inducing ovulation in some anovulatory mares during spring transition or during the breeding season, the tremendous variability observed in both pituitary and ovarian responses among mares suggests that other limitations within the hypothalamic-pitutiary axis are operable.

Gonadotropin Inhibiting Hormone

Recently, the first endogenous hypothalamic peptide found to inhibit gonadotropin release in a vertebrate was reported (75). This dodecapeptide, called gonadotropin inhibiting hormone (GnIH), has a C-terminal Leu-Pro-Leu-Arg-Phe-NH_2

sequence with an RF amide at the C-terminus (75). The cDNA encoding GnIH has been localized in several species of seasonally reproductive avian species (75-79), and the diencephalon has been shown to be the primary site of expression of the GnIH gene (75, 76). Gonadotropin inhibiting hormone-like immunoreactive (GnIH-ir) cell bodies are also distributed throughout the diencephalic and mesencephalic regions, with an abundance located in the paraventricular nucleus of the hypothalamus (75, 79). However, precursor mRNA for GnIH was found to be expressed only in the paraventricular nucleus, which indicates that the PVN is the only site for synthesis of GnIH (79). Also, RF amide-related peptide-1 (RFRP-1) and RF amide-related peptide-2 (RFRP-2) have been purified from rat, bovine and human hypothalamic tissue (77, 78). Both of these peptides have a very similar sequence to GnIH, indicating that they may have evolved from the same ancestor.

In seasonally-breeding birds, GnIH first begins to function around the time of hatch (80). *In vitro*, GnIH has been shown to inhibit LH release from quail anterior pituitary in a dose-dependent manner, but there was no effect on FSH or prolactin release (75). In adult chickens, addition of GnIH to pituitary cultures caused a decrease in FSH and LH release (81). Similarly, injection of synthetic GnIH into white-crowned sparrows resulted in a rapid decrease in plasma LH (82). In addition, GnIH-ir neurons, which are localized in multiple brain locations, exhibit larger areas at the end of the breeding season than at other times (83). These studies confirm that GnIH has *in v*itro, as well as *in vivo*, anti-gonadotropic functions in both laboratory and field settings.

Recently, Ubuka et al. (84) have shown that melatonin produced from the pineal gland and eyes induces the release of GnIH in quail. Pinealectomy combined with orbital enucleation caused a concomitant decrease of GnIH precursor mRNA, GnIH peptide and endogenous melatonin. Likewise, administration of melatonin to pinealectomized and orbital enucleated birds increased GnIH mRNA expression and GnIH concentration in a dose-dependent manner. In addition, GnIH mRNA and GnIH peptide have been shown to be elevated during short days, when melatonin secretion is known to be highest (84). Collectively, these findings suggest that GnIH has a major role in controlling seasonal reproduction in photoperiodic avian species and may provide insight into the possibility of a similar control mechanism in the equine.

CHAPTER III

EVIDENCE THAT THE ATTENUATED RELEASE OF LH ACCOMPANYING ANOVULATORY AND TRANSITIONAL SEASONS IN MARES OCCURS WITHOUT COINCIDENT SUPPRESSION OF PULSATILE GnRH SECRETION

Introduction

Mares are seasonal long-day breeders with a loosely defined natural breeding season that extends from April to October in the Northern Hemisphere. Approximately 80-85% of mares have been reported to cease ovarian cyclic activity at some time during the fall and winter months (1). Although not well-characterized in horses, the control of seasonality appears to involve changes in pineal melatonin secretion that modify the reproductive neuroendocrine axis through multiple neuronal pathways. During fall transition, the end result is cessation of ovulatory cycles and can be accounted for by a marked reduction in the synthesis and release of anterior pituitary LH (26, 29, 30). Based upon several lines of limited evidence in horses (22, 60, 64, 73), and studies in other seasonal mammals such as the ewe (74), it is generally assumed that lack of an adequate hypothalamic-derived GnRH signal to the anterior pituitary drives this transition (1). However, this has not been clearly demonstrated in well-controlled studies using methodologies capable of measuring the acute, pulsatile secretion of GnRH.

Owing to its low concentration and rapid metabolism, the actual *in vivo* measurement of GnRH secretion in the peripheral circulation is not possible. Therefore, several techniques have been established for the measurement of GnRH secretion in mammals. Although not widely exploited, a unique procedure for collecting pituitary venous effluent from the anterior pituitary of mares via cannulation of the intercavernous sinus (ICS) was reported in the early 1980's (65). This approach is possible only in equids due to the unique arrangement of their cranial and facial vasculature, and allows the coincident measurement of pulsatile LH and GnRH secretion. In the current study, we used the ICS cannulation technique to revisit the hypothesis that marked reductions in secretion of LH during transition into and out of the ovulatory season, as well as during the anovulatory season can be accounted for by similar reductions in hypothalamic GnRH secretion.

Materials and Methods

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

Animals

Eleven maiden or barren Quarter Horse mares were obtained from a local breeder, Flying V Quarter Horses, in mid-June, 2004. Mares were maintained on pasture and supplemented as needed with Coastal bermudagrass hay and concentrate (14% crude protein, mixed grain, soybean meal and molasses; Falls City Milling, Falls City, TX) to achieve and maintain a body condition score of 5-6 (85).

Throughout the study (July 2004 – May 2005), the estrous cycles of all mares were monitored through daily teasing with a stallion and periodic ultrasonography of ovarian structures. Teasing scores were as follows: 1) breaks down, winking, urinates, 2) interest in stallion, 3) passive, 4) rejects stallion. The reproductive tracts of all mares were examined using transrectal ultrasonography (Concept/MCV, Dynamic Imaging, Livingston, Scotland, UK) three times weekly except when visually observed in estrus or having a follicle of $\geq$ 35 mm, at which time ultrasound examinations were performed every other d until ovulation was confirmed. Blood samples were collected via jugular venipuncture at the time of every ultrasound examination. Samples were placed on ice immediately following collection. After transfer to the laboratory, they were allowed to clot at room temperature for 45 min to 1 h before collection of serum by centrifugation. Jugular samples were stored at –20° C until hormone analysis for concentrations of progesterone.

Experimental Procedures

Objectives were to cannulate the intercavernous sinus (ICS) of 11 mares via the facial veins for intensive blood sampling at four distinct times during the calendar year: Ovulatory Season (August 10-23); Fall Transition (November 9-30); Winter Anovulation (January 19 – February 1); Spring Transition (March 24 – May 24). Due to potential complications associated with repeated cannulation of the same vein, (Dr. Dan Sharp, University of Florida, personal communication; unpublished observations from this laboratory), we employed a strategy that, in a worst-case scenario, would insure our ability to collect intensive samples at a minimum during the ovulatory season and winter

anovulation. Thus, we arbitrarily assigned the right facial vein for cannulation and sampling during the ovulatory and fall transition periods and the left facial vein for sampling during the anovulatory and spring transition sampling periods. During the fall transition cannulations, which were the first recannulations of the study, we did experience difficulty in locating the vein due to large amounts of scar tissue and in threading the catheter up the vein, presumably due to sclerosis. However, with experience, we were able to overcome this obstacle and had no cannulation failures in subsequent seasons. In addition, we experienced difficulties observed previously (22), during several cannulations in which the catheter entered a venous branch that traveled rostrally toward the nostril (Figure 1). This problem was usually easy to overcome by readjusting the entry site or manipulating the catheter until the desired positioning was achieved. Depending upon cannulation success and cannula function, the total number of mares sampled successfully during each season varied from a maximum of 11 during the ovulatory season, winter anovulation and spring transition to a minimum of eight during fall transition. However, intensive samples were collected successfully from only seven mares during all four seasons and only data from these mares were included in the final analysis (n = 7).

During the ovulatory season, intensive samples were collected during the follicular phase when the dominant, preovulatory follicle reached approximately 35 mm and estrous behavior was observed. All mares ovulated within 120 h following intensive sampling except one mare (mare 7), who ovulated less than 24 h before sampling. We chose to include data from Mare 7 because it has been established that the ovulatory

surge does not terminate until 24-48 h following ovulation (1). Mean follicle size at the time of sampling was 36.8 ± 1.67 mm.

Intensive sampling during fall transition into the anovulatory season was performed approximately at a midpoint between the fall equinox and winter solstice. Retrospective analyses of physiological status of mares during this period revealed three ovarian physiological categories: 1) absence of significant follicular growth and anovulatory, n=2; 2) follicle growth to at least 35 mm, estrous behavior, but failure to ovulate, n=3; 3) ovulatory, n=2. One mare (mare 44) in category 3 ovulated within 5 d after fall transition sampling, and this represented her last ovulation of the year. The other mare (mare 4) in category 3 ovulated within 48 h following fall transition sampling and continued to exhibit cyclic ovarian activity until her final ovulation in mid-January.

Sampling during winter anovulation was performed when all mares had ceased ovulating, approximately 1 mo following the winter solstice. As noted above, mare 4 had her final ovulation on January 14; therefore, intensive sampling of this mare was performed on February 1, allowing time for the anovulatory state to be confirmed.

Intensive blood sampling during spring transition into the ovulatory season was systematically performed immediately before the second ovulation of the year in each mare. As in the ovulatory season, intensive samples were collected when the dominant, preovulatory follicle reached approximately 35 mm and estrous behavior was observed. All mares ovulated within 6 d following sampling, and mean follicle size at time of sampling averaged 38.4 ± .74 mm.

Cannulation of the ICS was performed in each mare 18 - 24 h before the collection of intensive samples. On the d of sampling, mares were heparinized (30,000 IU heparin iv), tied loosely, and provided with hay and water throughout the blood collection procedure. Five-ml blood samples were collected at 5-min intervals for 8 h. Samples were placed into tubes containing 50 µl of a 5% EDTA- heparin solution (10,000 IU/ml) to prevent coagulation. Collection tubes also contained 100 µl of a 50 mM solution of bacitracin (Sigma Chemical Co., St. Louis, MO, USA) to minimize metabolism of GnRH. Samples were placed immediately on ice and centrifuged (5125 x g) for collection of plasma every 15 – 30 min. Plasma was stored at –80° C until hormone analysis for concentrations of GnRH, LH and FSH. At the end of each intensive blood collection period, the cannula was removed and each mare was returned to the herd. The skin overlying the site of cannulation was inspected daily and treated with a topical antiseptic as required until healed.

Intercavernous Sinus Cannulation Procedure

Mares were placed in a stock and sedated with Dormosedan (20-40 µg/kg BW; Pfizer Animal Health, Exton, PA). The facial vein was located by palpation of an area parallel to and along the anterior border of the mandible. An area centered over this line and extending in all directions for approximately 2 – 2.5 cm was clipped, scrubbed and disinfected for aseptic surgery using an iodophore. An area (approximately 1 cm x 1 cm) overlying and surrounding the vein, but below the facial crest, was infiltrated sc with Lidocaine HCl (2%; Vedco, St. Joseph, MO). A 1-cm skin incision was made over the vein, and the vein was exteriorized with blunt dissection and held in the exteriorized

position by placing a sterile probe between it and the underlying tissue. A small incision was made in the vein and a Tygon catheter (S-54-HL, ID .040", OD .070", Norton Performance Plastics Corporation, Akron, OH) bearing a stainless steel, flexible stylette, was inserted into the vein. The catheter was then gently threaded caudally and toward the cranium into the ICS (Figure 1). Once in place, the stylette was removed and a heparin (10,000 IU/ml) lock was placed in the tubing. Tubing was secured with synthetic polyamide sutures (Supramid, S. Jackson, Inc, Alexandria, VA) above and below its exit through the skin, and the skin was partially closed and dressed with an antiseptic skin dressing. A lateral radiograph was taken to visualize the position of the catheter and verify that it was located in the ICS (Figure 2). During sampling, each mare was heparinized (30,000 IU heparin every 3 h) and blood samples were collected manually with minimal negative pressure (3-5 cc syringe). For this experiment, catheters remained in place for no longer than 2 d, and were removed immediately following each intensive sampling period. Each mare was given parenteral antibiotics (procaine penicillin, 1,363 IU/kg BW) on the day of surgery and intensive sampling. If abnormal swelling occurred around the site of cannulation, antibiotics were administered for an additional 3 d.

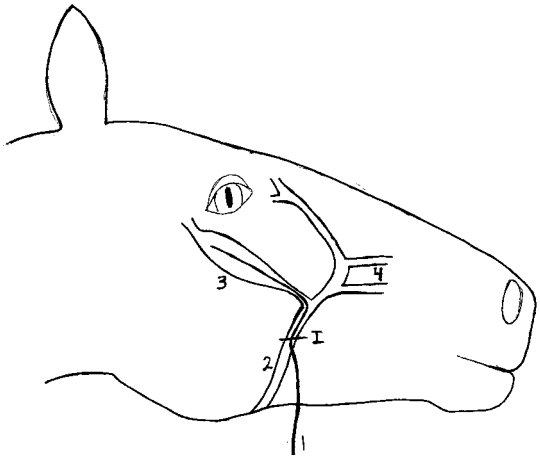

Figure 1. Drawing of the cranial vasculature of the horse showing the path followed by the cannula into the intercavernous sinus. The cannula insertion site is marked by the letter I. 1. Cannula, 2. Superficial facial vein, 3. Intercavernous sinus, 4. Superficial nasal veins

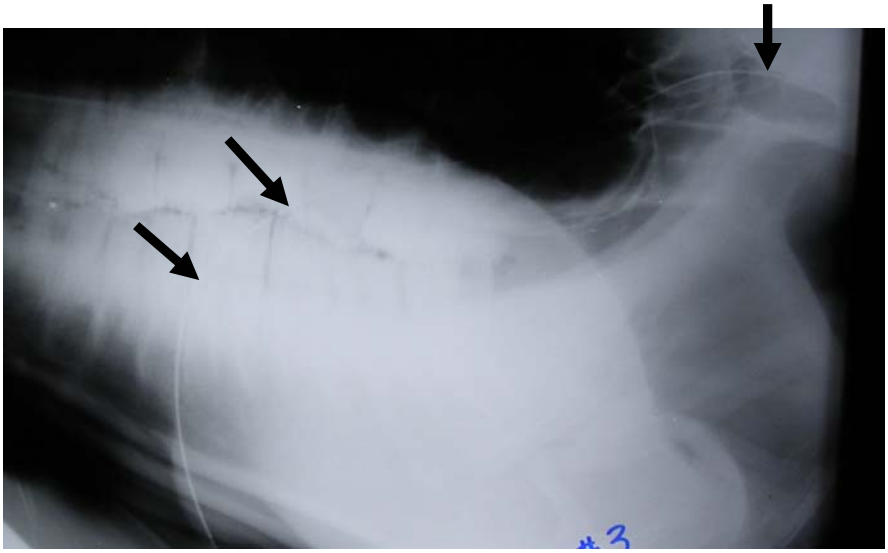

Figure 2. Lateral radiograph post-cannulation. The arrows help identify the cannula, with the guide wire still in place as it travels toward the cranium into the ICS, with the tip resting just below the orbit of the eye.

Hormone Analysis

Plasma concentrations of GnRH for all intensive samples collected were determined by RIA as described by Ellinwood et al. (86), with other modifications described in detail elsewhere (87). Antiserum BDS 037 (Dr. Alain Caraty, Nouzilly, France) was used as the source of first antibody at a final dilution of 1:150,000. The sensitivity of the assay, and intra- and interassay coefficients of variation were 1 pg/ml, 18.8% and 20.5 %, respectively.

Plasma concentrations of LH and FSH were determined by double antibody RIA for all intensive samples collected. A highly purified equine LH (eLH AFP-5130A) preparation was used for both iodinated tracer and standards. An anti-eLH antiserum (AFP-240580) was used at a dilution of 1:120,000. A highly purified equine FSH (eFSH AFP-5022B) preparation was used for both iodinated tracer and standards. An anti-eFSH antiserum (AFP-2062096) was used at a dilution of 1:12,500. The sensitivities, intra- and interassay coefficients of variation were 0.1 ng/ml, 8.9% and 10.7%, respectively for LH, and 0.5 ng/ml, 9.3% and 15.9%, respectively for FSH.

Serum concentrations of progesterone were measured using a commercial RIA kit (Diagnostic Products Corporation, Los Angeles, CA) for all jugular samples collected as reported previously from this laboratory (88). The sensitivity, intra- and interassay coefficients of variation were 0.1 ng/ml, 9.9% and 10.9 %, respectively.

Pulse Analysis

As noted previously by Irvine and Alexander (22), GnRH secretion as measured in the ICS in the mare appears to occur in a nearly continuous manner, with major

episodes of secretion often punctuated by multiple pulses of high frequency. However, subjective evaluation of LH and GnRH pulse patterns suggested that LH pulses were associated more closely, as would be expected, with the major episodes of GnRH release and not necessarily each individual peak or pulse. This phenomenon can be observed in Figure 1 where Mare 5 exhibited 15 pulses of LH, markedly fewer than the number of GnRH peaks or pulses observed subjectively. Therefore, we examined the effect of transforming hormone data (GnRH, LH, FSH) from samples collected at 5-min intervals using a 10-min rolling average to reduce background noise, particularly for GnRH. Putative pulses were then subjectively identified by three separate individuals and a final consensus developed using a pulse detection algorithm (Pulsefit 1.2). Figure 3 shows a comparison of hormone data plotted for Mare 5 using values from both 5-min samples and those generated using a 10-min rolling average. This process had little effect on the frequency of LH pulses, but reduced the number of pulses or episodes of GnRH secretion to a number that more closely resembled that for LH and resulted in a high degree of correspondence (overall correlation coefficient for GnRH and LH pulses was 0.65; $P < 0.001$ and 75% of all GnRH pulses corresponded with an LH pulse) between GnRH and LH pulses. Therefore, this approach was utilized for final pulse detection analyses for all hormones.

Statistical Analysis

Only mares for which complete data from all four seasons were obtained were included in the statistical analysis (n=7). Data were analyzed using the GLM procedure of SAS (SAS Inst., Inc., Cary, NC) to determine the effect of season on frequency and amplitude of pulses and mean concentrations of GnRH, LH and FSH. Three methods were used to determine differences in mean concentrations of each hormone: 1) Least squares means were obtained from raw data (96 values/mare/season) and analyzed using mare (season) as the error term to minimize individual mare variation. 2) Least squares means were converted to percent change using the mean of the ovulatory season as time zero, or 100%. Values were then analyzed with one-way ANOVA to test for effects of season. 3) Least squares means for each mare for each season were calculated and means were analyzed with one-way ANOVA to test for effects of season. Results of each of these analyses produced similar results. Mean concentrations of LH, GnRH and FSH are presented as described in 3 above.

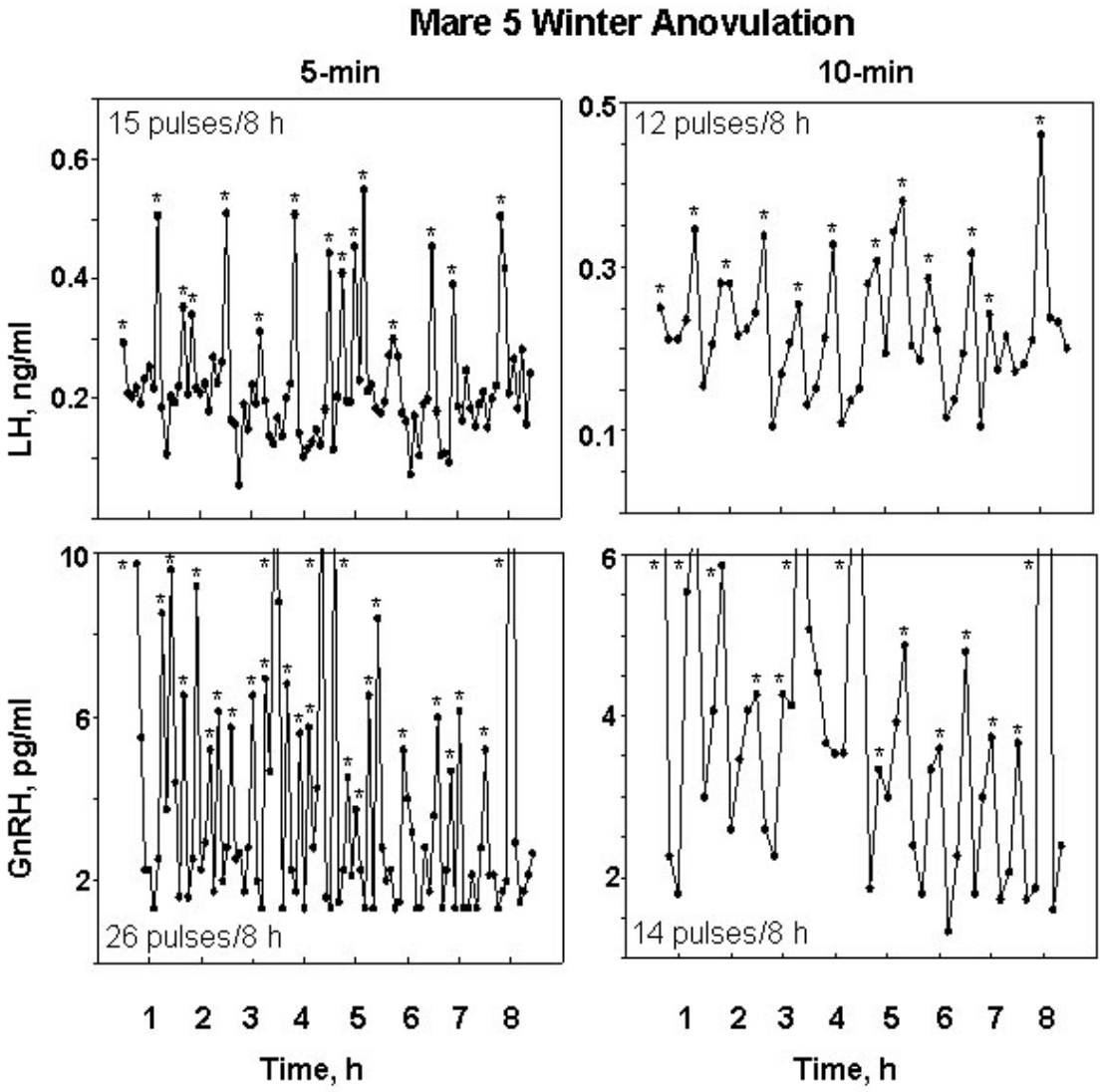

Figure 3. Five-min (left panels) and transformed 10-min (right panels) secretory patterns of GnRH (bottom) and LH (top) in one representative mare (Mare 5) during the winter anovulatory sampling period.

Results

Figure 4 presents least squares mean concentrations of LH, FSH and GnRH during the ovulatory season, fall transition, winter anovulation and spring transition. Concentrations of LH were markedly lower ($P < 0.01$) during fall transition, winter anovulation and spring transition compared to the ovulatory season. Following fall transition sampling, two mares ovulated and five did not. The two ovulatory mares had greater ($P < 0.01$) mean concentrations of LH (1.92 ± 0.02 ng/ml) and lower ($P < 0.01$) mean concentrations of FSH (4.76 ± 0.33 ng/ml) compared to the 5 anovulatory mares (0.34 ± 0.02 and 8.69 ± 0.21 ng/ml, for LH and FSH, respectively). Mean concentrations of GnRH were not affected by cycling status at fall transition sampling. As mares

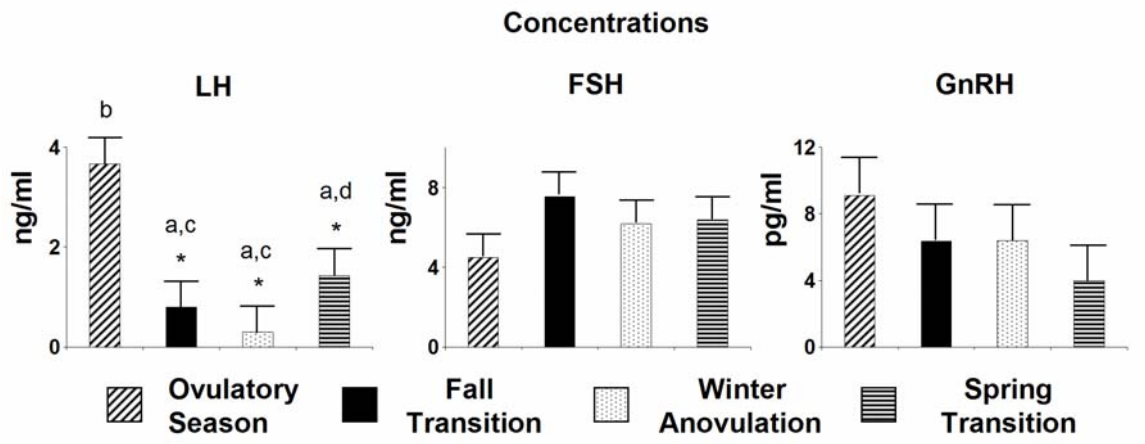

Figure 4. Least squares mean (± SEM) concentrations of plasma LH, FSH and GnRH as determined in ICS blood samples collected at 5-min intervals from seven mares during the ovulatory season, fall transition, winter anovulation and spring transition. Means with different superscripts differ (^{a,b} $P < 0.01$; ^{c,d} $P < 0.05$).

returned to ovarian cyclicity during spring transition, mean concentrations of LH increased and were greater ($P < 0.05$) than during the winter anovulatory season. However, changes in mean concentrations of LH were not accompanied by corresponding changes in GnRH and the latter did not differ due to season. In addition, there was no effect of season on circulating concentrations of FSH.

Mean amplitudes of pulses of LH, FSH and GnRH during each sampling period are summarized in Figure 5. The marked reduction in concentrations of LH during fall transition, winter anovulation, and spring transition compared to the ovulatory season coincided with a similar reduction ($P < 0.01$) in the amplitude of LH pulses. Moreover, similar to that observed for concentrations of LH (Fig. 4), an increase ($P < 0.05$) in amplitude of pulses of LH was observed during spring transition compared to the winter

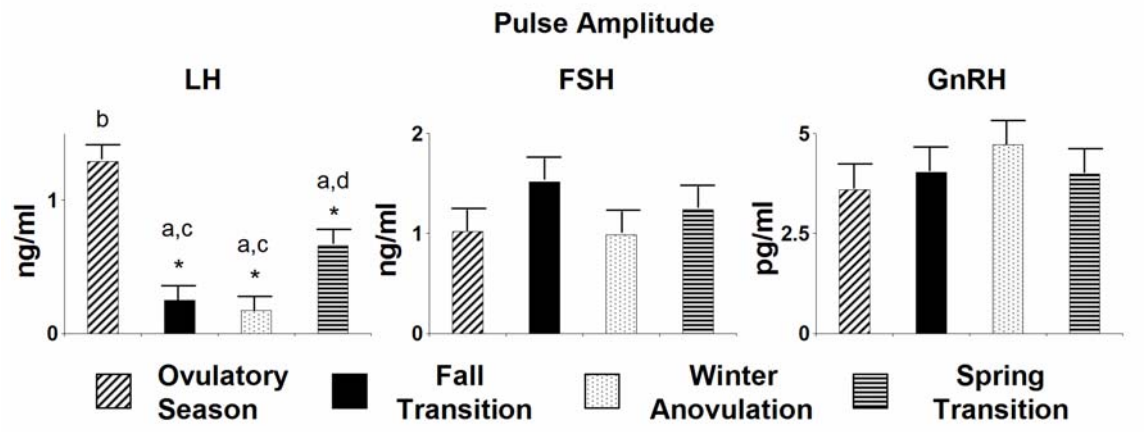

Figure 5. Least squares mean (± SEM) amplitudes of pulses of LH , FSH and GnRH as measured in ICS samples collected at 5-min intervals from seven mares during the ovulatory season, fall transition, winter anovulation and spring transition. Means with different superscripts differ (^{a,b} $P < 0.01$; ^{c,d} $P < 0.05$).

anovulatory season and fall transition. However, as observed earlier for concentrations, changes in the amplitude of LH pulses were not accompanied by corresponding changes in the amplitude of GnRH pulses and the latter did not differ due to season. Similarly, the mean amplitude of FSH pulses was not affected by season.

Figure 6 summarizes the mean frequencies of pulses of LH, FSH and GnRH during the study. While concentrations and amplitudes of pulses of LH were affected markedly by season (Figures 2 and 3), there were no effects of season on the frequency of LH pulses. Correspondingly, season did not affect the frequency of GnRH pulses. However, the frequency of FSH pulses was slightly lower ($P < 0.03$) during the fall transition compared to all other seasons.

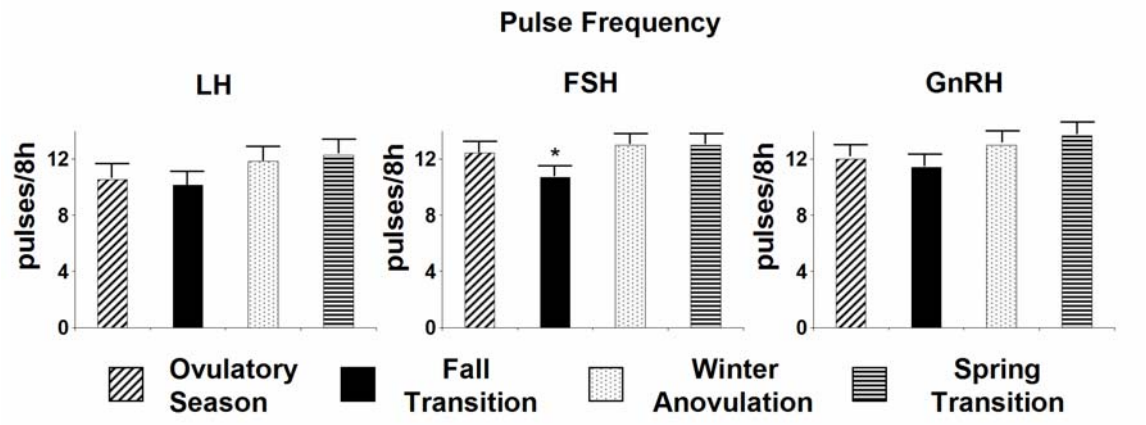

Figure 6. Least squares mean (± SEM) frequencies of pulses of LH, FSH and GnRH as measured in ICS samples collected at 5-min intervals from seven mares during the ovulatory season, fall transition, winter anovulation and spring transition. * denotes a difference ($P < 0.03$)

Patterns of pulsatile GnRH, LH and FSH secretion for 3 representative mares are presented during the ovulatory season (Figure 7), fall transition (Figure 8), winter anovulation (Figure 9) and spring transition (Figure 10), respectively. These mares were elected for depiction because they each represented a different physiological status during sampling at fall transition into the anovulatory state. The overall correlation coefficient for GnRH and LH pulses was 0.65 ($P < 0.001$) and 75% of all GnRH pulses corresponded with an LH pulse. Similarly, the overall correlation coefficient for GnRH and FSH pulses was 0.55 ($P < 0.002$) and 73% of all GnRH pulses corresponded with an FSH pulse. This is similar to what was expected, as it is generally accepted that not every GnRH pulse produces a pulse of LH or FSH. In addition, the overall correlation coefficient for LH and FSH pulses was 0.33 ($P < 0.08$).

During the ovulatory season (August 12-23; Figure 7), each of the three representative mares were exhibiting normal estrous cycles and ovulated within 48 h after sampling. However, Mare 4100 had a final ovulation on September 2 and was anovulatory at the time of fall transition sampling (Category 1, November 21; Figure 8). Following her final ovulation, the mare developed a persistent CL, resulting in chronically-elevated levels of progesterone. Preceding targeted intensive sampling for fall transition on November 8, 25 mg prostaglandin $F_{2\alpha}$ was administered to cause regression of the CL. No follicular activity was observed within 2 wk after injection and luteal regression, and the mare was therefore considered anovulatory. Mare 16 had a final ovulation 23 d before the fall sampling period (November 24). At the time of fall transition sampling, this mare had developed what appeared to be a normal (35 mm)

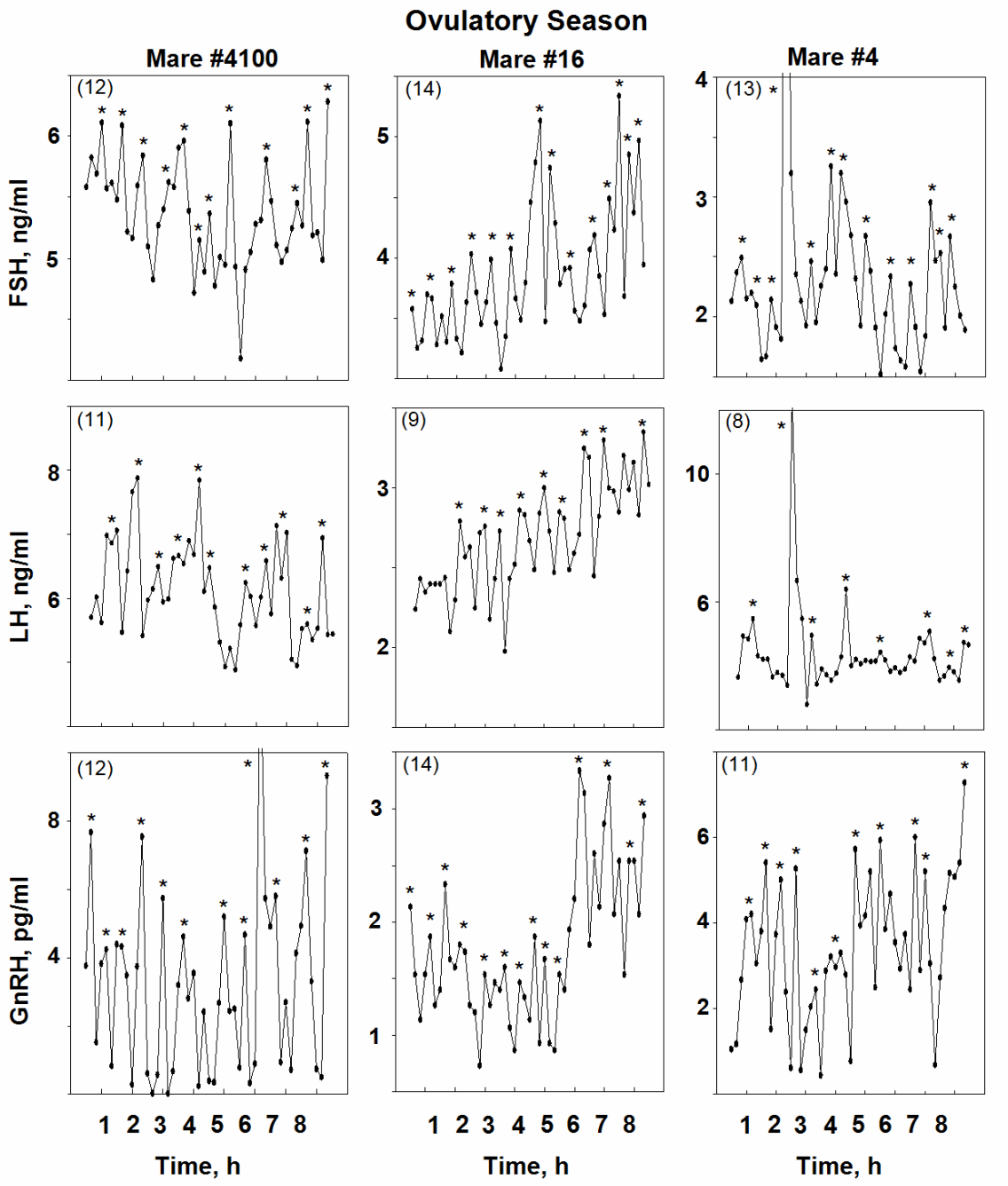

Figure 7. Patterns of GnRH, LH and FSH secretion in three representative mares during the ovulatory season. Pulses are denoted by an asterisk.

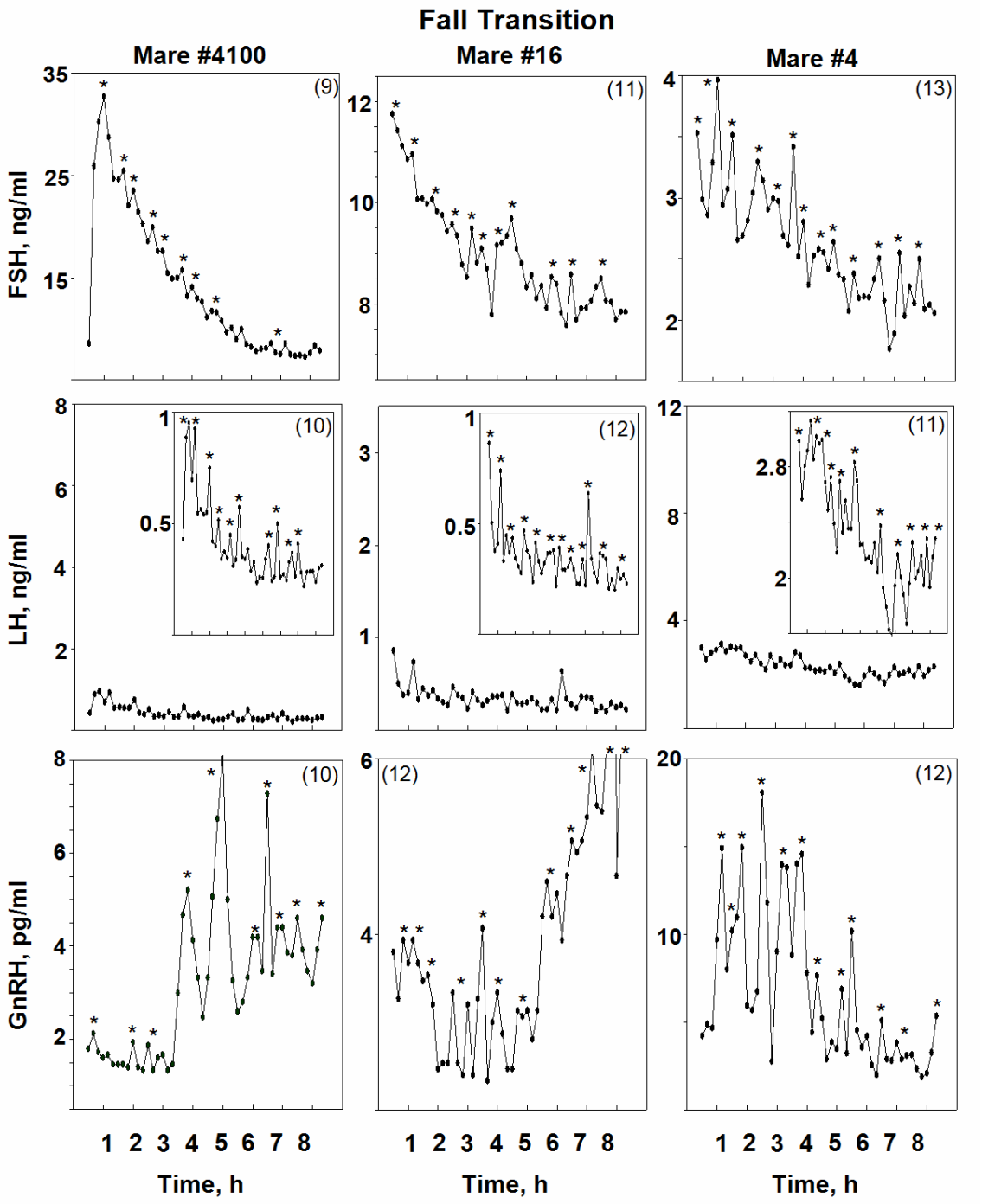

Figure 8. Patterns of GnRH, LH and FSH secretion in three representative mares during fall transition out of the ovulatory season. Pulses are denoted by an asterisk

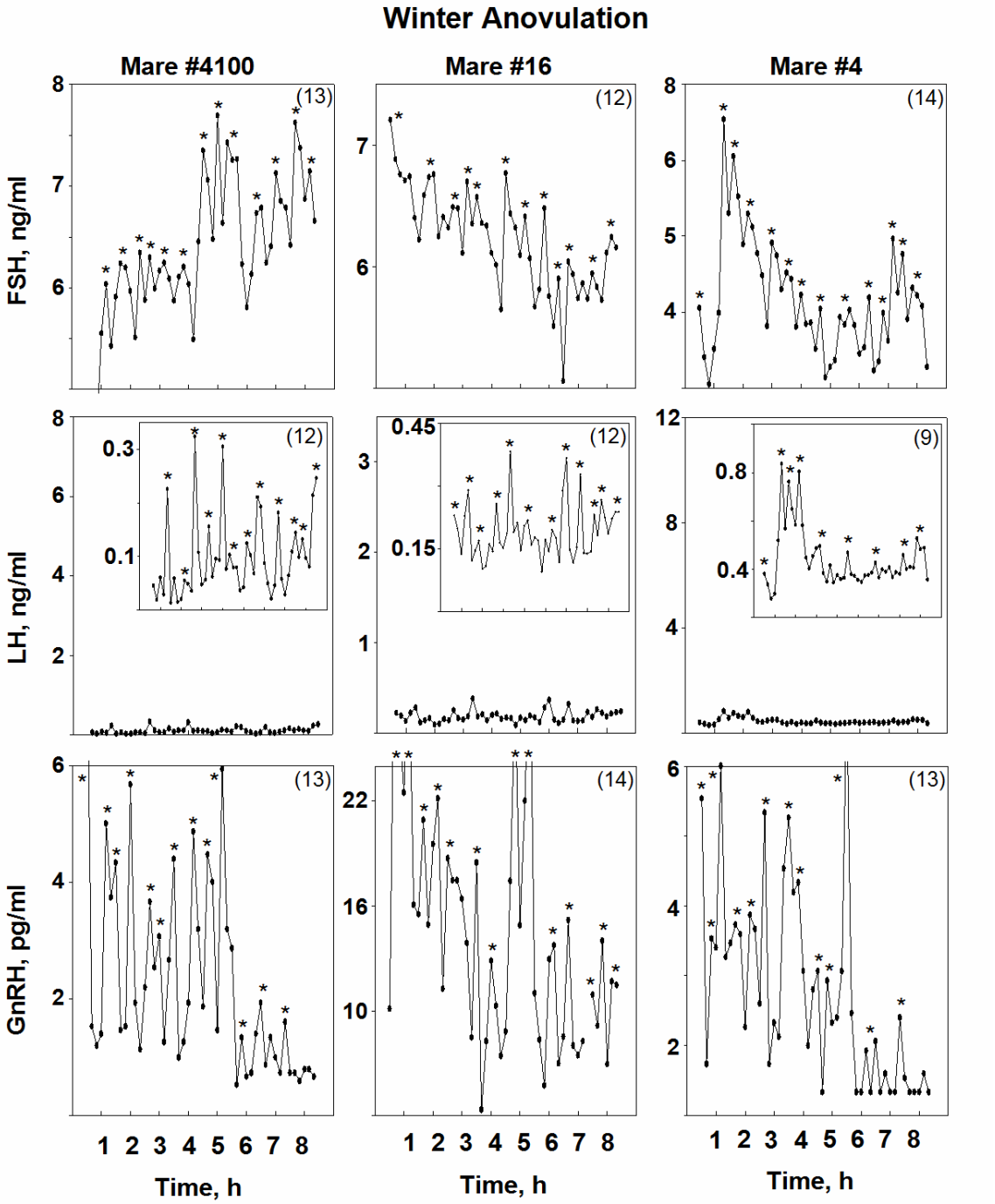

Figure 9. Patterns of GnRH, LH and FSH secretion in three representative mares during winter anovulation. Pulses are denoted by an asterisk.

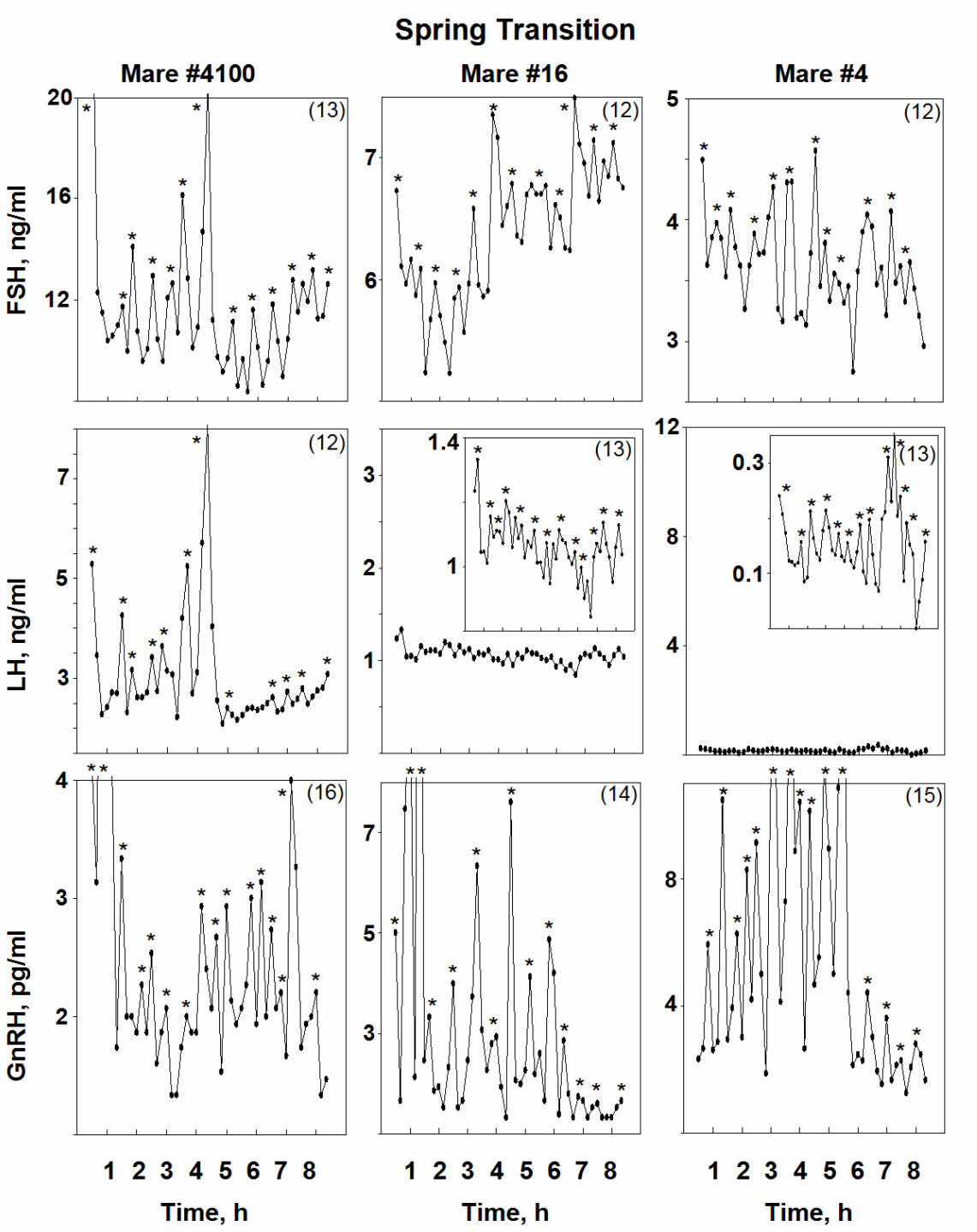

Figure 10. Patterns of GnRH, LH and FSH secretion in three representative mares during spring transition into the ovulatory season. Pulses are denoted by an asterisk.

preovulatory follicle and showed estrous behavior. However, the mare failed to ovulate following sampling (Category 2; Figure 8). Mare 4 ovulated 24-48 h following fall transition sampling (category 3), and had a final ovulation on January 14.

As observed for the three representative mares, pulsatile patterns of FSH, LH and GnRH were clearly evident for all mares during both the ovulatory season and fall transition, with the LH baseline markedly higher during the ovulatory season compared to the fall. The marked reduction in mean concentrations of LH in November compared to August, which occurred in all mares in this study, was associated with a similar diminution of the amplitudes of individual pulses of LH. However, pulse frequency was not affected. This relationship is easily visualized by evaluating the pattern of LH secretion on the expanded scales (insets) during fall transition (Figure 5). Further inspection of Figures 7, 8 and 9 reveals baseline concentrations of GnRH secretion that for Mare 16 were actually greater during the fall transitional period and winter anovulation than during the ovulatory season. This observation further supports the interpretation that the decline in mean circulating LH between the two seasons can be accounted for by a diminishing (but not absent) ability of the anterior pituitary to respond to individual pulses of GnRH and not to a reduction in the frequency or amplitude of GnRH pulses.

A unique, surge-like pattern of FSH release was observed for several mares, primarily during fall transition sampling (Figure 8). The corresponding decrease in mean frequency of pulses of FSH at this time (Figure 4) appears to be a result of this pattern of hormone release. The pattern mimicked that observed during the preovulatory

gonadotropin surge in other mammals (see Discussion) and consisted of an exponentially-declining concentration after the peak and individual small pulses on the descending side. Concentrations of LH, although quite low, exhibited a similar peak and pattern of decline on the expanded scale (Figure 8). While frequency of pulses of GnRH generally duplicated that seen during all other seasons, including the ovulatory season, concentrations of GnRH did not follow the same pattern as LH and FSH during these episodes.

Figure 9 illustrates patterns of pulsatile GnRH, LH and FSH secretion for the three representative mares during winter anovulation (January 21 - February 1), when all mares had ceased the development of large follicles and ovulation. As during the ovulatory season and fall transition, pulsatile patterns of GnRH, LH and FSH were clearly evident during winter anovulation. In addition, the sustained decrease in mean concentrations of LH during both the fall and winter were not reflected by changes in the frequency of LH pulses. This can be seen clearly by evaluating the pattern of LH secretion on the expanded scales (insets) during both fall transition and winter anovulation for each individual mare (Figures 8 and 9).

Patterns of GnRH, LH and FSH secretion during spring transition into the ovulatory season are depicted in Figure 10. Pulsatile patterns of release for all three hormones were quite obvious and mean concentrations of LH were increasing at this time compared to the fall transition and winter anovulation periods (Figure 4). This phenomenon is visually apparent upon subjective inspection of two of the individual representative mares, including Mare 4100, in which concentrations of LH were great

enough to make pulses visible on the same scale as during the ovulatory season. While concentrations of LH also had increased for Mare 16 during spring transition, the pulses were much more evident when viewed on an expanded scale. Mean concentrations of LH for Mare 4 during spring transition remained at suppressed levels and were similar to those observed during fall transition and winter anovulation. Therefore, pulses must be viewed on an expanded to scale to be visually evident. The relatively large differences observed in mean concentrations of LH during spring transition for these individuals illustrate the relatively large variation in the LH baseline that can attend the demise and resumption of ovulatory cycles.

Baseline concentrations of GnRH varied, sometimes quite dramatically, among different mares within season. This can be observed by inspecting baseline values of the three representative mares during the winter anovulatory season (Figure 9). Mare 4100 and Mare 4 both had an approximate baseline concentration of GnRH of 2 pg/ml. However, the GnRH baseline for Mare 16 was approximately 10 pg/ml. This variation demonstrates the value of sampling the same individual mares throughout all four seasons.

Figure 11 presents representative patterns of serum progesterone in jugular samples collected two to three times weekly throughout the study (July - May) for the three individual mares discussed in Figures 7 through 10. Cyclicity for each mare was confirmed by the elevated progesterone values during the luteal phase following ovulation during the ovulatory season. Differences that existed in timing of fall transition and onset of winter anovulation among the individual mares are readily apparent. As discussed previously, Mare 4100 ended the ovulatory season with the formation of a persistent corpus luteum, which is clearly reflected by the sustained period of progesterone elevation seen in Figure 11 (top panel). Individual patterns also demonstrated that the length of the anovulatory period can be dramatically different from mare to mare. Mare 4100 and Mare 16 had similar durations of seasonal anovulation: 7 and 5.5 mo, respectively. However, Mare 4 ceased ovulation for less than 1 mo.

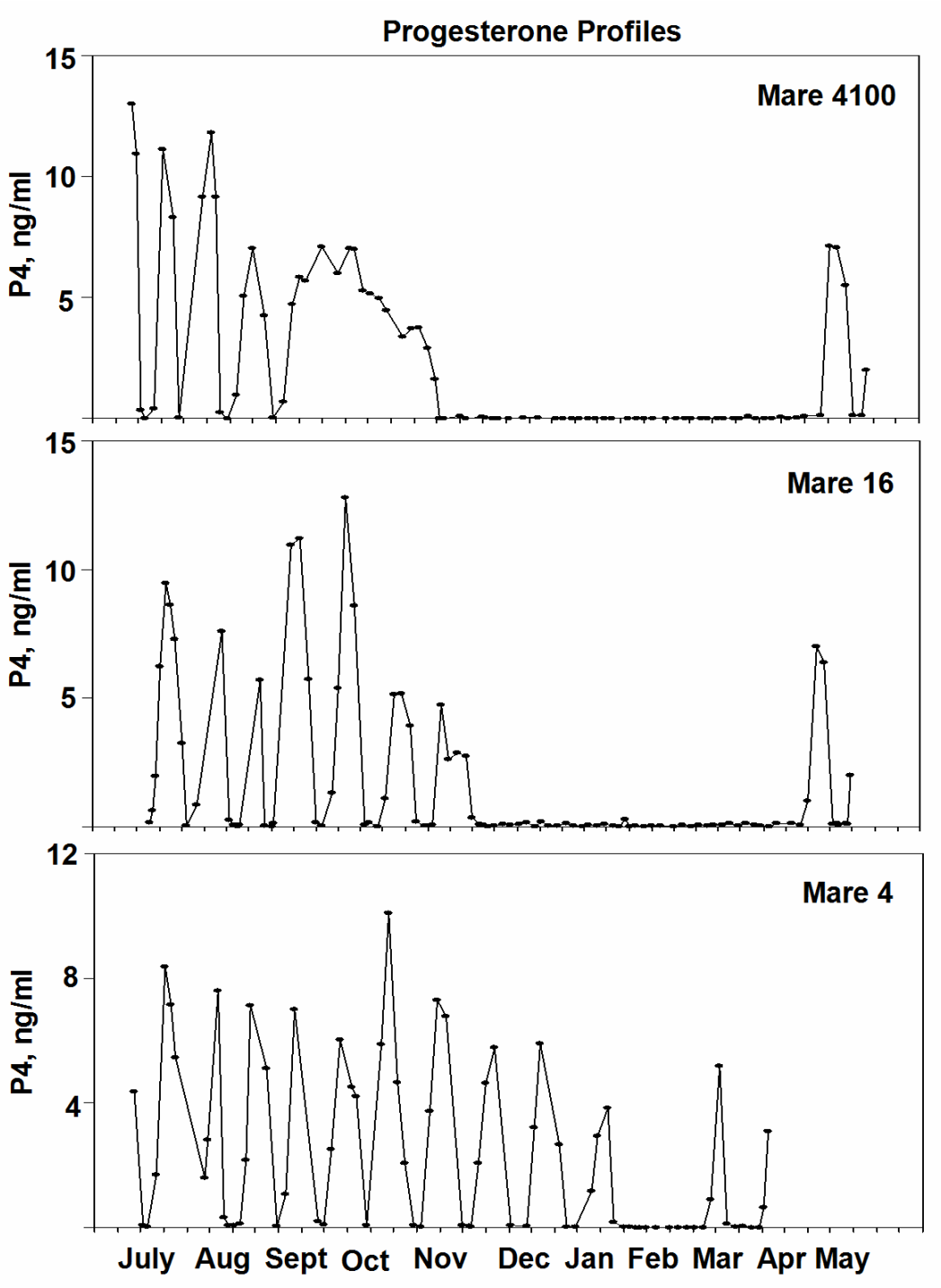

Figure 11. Patterns of progesterone concentrations measured in jugular samples collected two to three times weekly from July - May in three representative mares.

Discussion

Methods for assessing the pulsatile secretion of hypothalamic GnRH secretion in mammals during different physiological conditions have included direct cannulation of hypophyseal portal vessels in rodents, monkeys and sheep (89, 90, 91), cannulation of the third cerebroventricle in monkeys, sheep and cattle (91-93), and push-pull perfusion of hypothalamic tissue in situ in mares, sheep and rabbits (64, 94, 95). However, the unique vasculature of the ICS of the equine provides the potential for making similar measurements using a relatively non-invasive technique that is not possible in other mammals. Cannulation of the equine ICS as a method for monitoring the acute secretion of hypothalamic GnRH and anterior pituitary LH in the equine was first reported by Irvine and Hunn (65). However, this approach has not been widely exploited for that purpose and published reports have been limited to determining secretion of these hormones during different time points of the estrous cycle (67, 68, 72), with a paucity of data available on seasonality. Some of the challenges and complications associated with application of the procedure as outlined by Irvine and Alexander (22) were considered before embarking upon the study reported herein. Although these authors suggested that flow rate and other variables associated with the complexities of the ICS vascular compartment must be taken into account when using this approach to measure hormone secretion, the isotopic methodologies and mathematical calculations needed to address those issues make them impractical for routine studies. Thus, our approach was to standardize the cannulation procedure using radiographic comparison of catheter placement to sample from the same experimental animals repeatedly over time. Using

this approach, our objectives herein were to employ this procedure to at least semi-quantitatively describe and evaluate basic functional relationships between hypothalamic GnRH and anterior pituitary secretion of both LH and FSH during ovulatory, transitional and anovulatory seasons.

In the current study, measurement of GnRH, LH and FSH in samples collected from the ICS at 5-min intervals for 8 h during four seasons clearly demonstrate the ability to measure the pulsatile secretion of these hormones in the mare. During the ovulatory season, we found the secretion of these three hormones to be released, as reported previously, in relatively close temporal agreement (22, 64, 68). This is similar to the close temporal association characterized historically between GnRH and LH in numerous other mammalian species including sheep (94), cattle (93), monkeys (91), and rabbits (91) . Overall, we found that approximately 75% of GnRH pulses resulted in an LH pulse and 73% of GnRH pulses resulted in an FSH pulse. That every GnRH pulse does not result in an LH or FSH pulse has been documented previously in horses (22, 66, 67) and in other mammals (74, 90, 92).

The approach of winter solstice is accompanied by marked declines in circulating concentrations of LH and the onset of seasonal anovulation in the mare (30). The reduction in circulating LH has been shown to occur coincident with similar declines in anterior pituitary content of LH (55). Therefore, the decrease in mean concentrations of plasma LH observed during fall transition, winter anovulation and spring transition compared to the ovulatory season in the current study was as expected. However, contrary to accepted dogma, neither the frequency of LH pulses nor the frequency and

amplitude of GnRH pulses varied between seasons. The basis for large changes in circulating concentrations of LH during the transition into and out of the anovulatory season appears to be dramatic changes in the amplitude of individual pulses of LH. These are novel findings for several reasons. First, the use of the ICS cannulation technique to assess the pulsatile secretion of GnRH and LH in the same mares during the ovulatory season, fall transition, winter anovulation and spring transition has, to our knowledge, never been reported. Furthermore, in contrast to previous studies that employed push-pull perfusion of hypothalamic tissue to estimate GnRH secretion rates in different mares (64), or changes in the distribution (61) and content (54, 55, 60) of hypothalamic GnRH during different physiological states, our study examined the acute *in vivo* secretion of GnRH, LH and FSH in the same mares during different seasons.

The results of this study in the mare are in contrast to published reports of seasonal changes of GnRH secretion in the ewe (74). During the breeding season, ewes exhibit approximately 8 pulses of GnRH/6 h during the follicular phase of the estrous cycle, compared to < 1 pulse/6h during the anovulatory season (74). Therefore, it appears that, while the seasonal anovulatory state in ewes (a short-day breeder) is a result of the absence of a sustained volley of pulses of GnRH for driving secretion of LH, the basis for reduced LH in the seasonally anovulatory mare may reside at the level of the anterior pitutiary (ie, an inability of gonadotrophs to respond to the GnRH signal).

Results of data reported in this study provide additional insight into the basis for inconsistent responses and (or) failure of GnRH treatment to induce cyclicity in mares during the anovulatory period. If endogenous GnRH is not lacking during seasonal

anovulation, then it is understandable why exogenous treatment with this peptide would be unsuccessful in preventing winter anovulation or inducing cyclicity after it has begun (6, 10, 24). In fact, even in studies in which GnRH treatment successfully induced follicular development and ovulation with high frequency in transitional or idiopathic-anovulatory mares (23) only small increases in the LH baseline resulted from that treatment. In that study, circulating concentrations of LH remained 10 to 20-fold lower than observed in mares during the ovulatory season, yet 85 % of the mares developed large follicles and resumed ovulation. In the current study, while we found that mean concentrations of LH were greater for ovulatory than anovulatory mares during fall transition, the concentrations of LH in the ovulatory mares during fall transition were still dramatically reduced from the ovulatory season. The observation that mean FSH was lower in ovulatory compared to anovulatory mares can be speculated to be the result of a lack of negative feedback from the ovary that would still be present in ovulatory mares.

Several groups (31, 32, 33) have reported on the erratic nature of estrous behavior and ovarian events in mares during transition into the ovulatory season. Mares often have been shown to develop and to regress several follicles of preovulatory dimensions and exhibit prolonged estrous behavior prior to ovulating the first follicle of the season. Just as we could not predict the final ovulation in the fall, it was not possible to predict the first ovulation in the spring. Based on this information and personal experience, our objectives during spring transition were to sample each mare just before the second ovulation of the season in order to standardize physiological status. Mares in

the current study, as expected, exhibited lengthy and erratic periods of estrous behavior and the development of preovulatory size follicles that eventually regressed before the first ovulation.

Only one study has been published (73) concerning the pattern of secretion of GnRH and gonadotropins during spring transition. In that study, it was reported that all mares exhibited very infrequent or no pulses of GnRH, LH and FSH. Our results are in clear contradiction with that report, as all mares in the current study exhibited consistent patterns of GnRH, LH and FSH pulsatility during spring transition into the ovulatory season.

Our observations that FSH secretion changed little during different periods of the year are in agreement with one earlier report (29), but somewhat different than others (31, 35). In the latter reports, concentrations of FSH and amplitude of pulses were reported to decrease during spring transition. We observed no differences in mean concentrations and amplitude of pulses of FSH among all seasons; however, there was a slight but statistically significant decrease observed in frequency of pulses of FSH during fall transition compared to all other seasons. The appearance of surges of FSH, primarily during fall transition sampling (Figure 8), has been reported previously (71). The presence of surges during this period occurred coincident with a decreased frequency of individual pulses of FSH as noted previously. Surges of FSH were mimicked by similar patterns of LH, albeit at very low circulating concentrations (see expanded scales for LH in Figures 8-10), but were not associated with a corresponding temporal change in concentration of GnRH. The secretion of FSH is strongly influenced

by the stage of ovarian follicle development in mammals, and depending upon species, this influence often overrides acute effects of GnRH pulses on the pattern of FSH secretion. This is particularly true in cattle (96, 97). However, all reports to date using the ICS cannulation approach for detecting pulses in mares have demonstrated a close association between pulses of LH, FSH and GnRH (22, 64, 68). This association was clearly apparent in the current study, with a high correlation of individual pulses noted among all three of these hormones, even though temporal trends in baselines and mean concentrations for GnRH often did not follow that of the gonadotropins that it regulates. This was particularly true during periods when ovarian activity was compromised due to low LH. Further work will be required to more fully understand the regulation of FSH secretion in the mare during different seasons and to understand differences in the magnitude of changes in circulating gonadotropins compared to concentrations of GnRH in samples collected from the ICS.

Finally, although melatonin is involved in the process through which photoperiod regulates seasonal breeding, the precise signaling molecules involved in the regulation of gonadotropin secretion in seasonally-breeding animals are not known. However, new candidates have recently been discovered that could serve in this capacity and include gonadotropin-inhibiting hormone (GnIH) that functions at both the hypothalamic and pituitary level (75, 76, 79). Melatonin stimulates the synthesis of this neuropeptide in avian species within the paraventricular nucleus (84). Importantly, GnIH and related peptides have been identified in sheep (Dr. George Bentley, University of California, Berkeley, personal communication), cattle and rodents (77,78). Moreover, GnIH has

been shown to suppress LH secretion in photoperiodic avian species (81, 82), co-localizes with GnRH in the medial basal hypothalamus (82, 83), and has its own receptor on gonadotrophs (Dr. George Bentley, University of California, Berkeley, personal communication). One can only speculate as to the potential role of GnIH in seasonal reproduction in the mare. However, we have now identified GnIH in the equine hypothalamus and demonstrated co-localization with GnRH neurons in the medial basal hypothalamus at the level of the hypophyseal portal system (Williams et al. unpublished). Additional physiological, cellular and molecular studies will determine whether GnIH can account for the abrupt changes in gonadotroph responsiveness observed in the mare during different seasons of the year.

CHAPTER IV

CONCLUSIONS

Contrary to prevailing views, our results do not support the contention that the basis of declining synthesis and secretion of anterior pituitary LH is a reduction in the frequency and amplitude of GnRH pulses, nor in the amount of GnRH reaching the anterior pituitary. Data further imply that photoperiodic signals regulating seasonal breeding in the mare are comprised, at least in part, of inhibitory cues that directly affect the ability of gonadotrophs to respond to GnRH. The nature of such cues remains to be elucidated. However, it is tempting to speculate that they may include involvement of recently-discovered hormones (gonadotropin inhibitory hormone, GnIH; 75, 76, 79) and related peptides (77,78) that directly suppress the secretion of LH and pituitary responsiveness to GnRH in photoperiodic avian species (75,76,81,82). Further work will be required to examine these and other possibilities.

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APPENDIX

PROCEDURES

Equine FSH RIA

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A.F. Parlow, National Hormone and Peptide Program
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2. Antibody: Anti-equine FSH (AFP-2062096). Dilution 1:12,500.

3. Standards: Iodination grade eFSH (AFP-5022B). Range: 0.5 - 25.0 ng/ml.

4. References: eFSH added to equine serum.

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 withour 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 ^{125}I-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. Vortex tubes briefly and place in refrigerator for 48-72 h at 4°C.

C. Day 4: Take Off Assay

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

Equine LH RIA

References:

Davis, S.L., Riechert, L.E. and Niswender, G.D. Biol. Reprod. 4:415- (1971)
Echternkamp, S.E., Bolt, D.J. and Hawk, H.W. J. Anim. Sci. 42:893- (1976)
Golter, T.D., Reeves, J.J., O'Mary, C.C., Arimura, A. and Schally, A.V. J. Anim. Sci. 37:123- (1973)
Niswender, G.D., Riechert, L.E., Midgley, A.R. and Nalbandov, A.V. Endocrinology 84:1166- (1969)
Williams, G.L. and Ray, D.E. J. Anim. Sci. 50:906- (1980)

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

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

3. Standards: Iodination grade eLH (AFP-5130A). Range 0.1 – 20.0 ng/ml.

4. References: eLH added to equine serum

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-eLH (diluted in PBS-EDTA + 1:400 NRS) into all tubes except NSB and TC tubes.
   8. Pipette 100 µl ^{125}I-eLH (20,000 cpm/100 µl diluted in 1% PBS-EW) to 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 GnRH RIA

References:

Gazal, O. S., L. S. Leshin, R. L. Stanko, M. G. Thomas, D. H. Keisler, L. L. Anderson, and G. L. Williams. 1998. Gonadotropin-releasing hormone secretion into third-ventricle cerebrospinal fluid of cattle: Correspondence with the tonic and surge release of luteinizing hormone and its tonic inhibition by suckling and neuropeptide Y. Biol. Reprod. 59:676-683.

A. Extraction Procedure

1. Pipette 750 µl of plasma into borosilicate glass tube; (same for references).
2. Add 2 ml methanol.
3. Vortex briefly.
4. Centrifuge at 4°C, 300 rpm for 20 min.
5. Pour supernatant into polyethylene tubes.
6. Dry in apparatus under air stream.
7. Add 750 µl of PBS 0.1% Gel to reconstitute.
8. Assay 200 µl in triplicates.

B. Estimation of Extraction Efficiency (Performed at the same time as above)

1. Pipette 750 µl of peripheral pooled plasma/serum into 4 borosilicate glass tubes (Recovery tubes R1-R4).
2. Add 1500 cpm GnRH tracer into R1-R4 tubes and 4 total count of recovery tubes (TCR1-TCR4).
3. Add 2 ml methanol to R1-R4 tubes.
4. Vortex tubes briefly.
5. Centrifuge at 4°C, 300 rpm for 20 min.
6. Pour supernatant into polyethylene tubes.
7. Dry in apparatus under air stream.
8. Add 750 µl of PBS 0.1% Gel to reconstitute.
9. Store TCR and R tubes at 4°C; count at end of assay.

Efficiency = mean R1-R4 / mean TCR1-TCR4

C. Assay

1. Iodinated Product: Iodination grade GnRH (Sigma Chemical Co.)
2. Antibody: Anti-GnRH (Caraty BDS-037). Dilution 1:150,000.
3. Standards: Iodination grade GnRH (Sigma Chemical Co.). Range 1 – 1000 pg/ml

4. References: GnRH added to equine peripheral pooled plasma/serum

5. RIA Procedure:

A. Day 1: Begin assay (Extraction has been completed)
1. NSB – 400 µl of PBS + 0.1% Gel
2. 0 Std - 400 µl of PBS + 0.1% Gel
3. Stds – 200 µl std + 200 µl of PBS + 0.1% Gel
4. Ref – 200 µl ref + 200 µl of PBS + 0.1% Gel
5. Unknown – 200 µl sample + 200 µl of PBS + 0.1% Gel
6. Pipette 50 µl of PBS-EDTA + 1:400 NRS without primary antibody into NSB tubes only
7. Pipette 50 µl of anti-GnRH (diluted in PBD-EDTA+1:400 NRS) into all tubes except NSB and TC
8. Vortex tubes briefly
9. Incubate for 24 h at 4°C

B. Day 2: Add tracer
1. Pipette 50 µl ^{125}I-GnRH (12,000 cpm / 50 µl diluted in PBS + 0.1% Gel) to all tubes
2. Vortex briefly
3. Incubate for 24 h at 4°C

C. Day 3: Pour off
1. Add 2 ml ice cold ETOH to all tubes except TC
2. Vortex briefly
3. Incubate for 1 h at 4°C
4. Centrifuge tubes for 30 min at 4°C at 3600 rpm
5. Decant supernatant
6. Count radioactivity of each tube using a gamma counter

Progesterone RIA

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

References:

Jones, E.J., Armstrong, J.D. and Harvey, R.W. J. Anim. Sci. 69:1607 – (1991)

Diagnostic Products Corporation Coat-A-Count Progesterone Kit, Los Angeles, CA

Simpson, R.B., Armstrong, J.D. and Harvey, R.W. J. Anim. Sci. 70: 1478– (1992).

1. Iodinated Product: Iodination grade hP4.

2. Antibody: Anti-human P4 coated tubes.

3. Standards: Human serum with added P4. Range 0.1 – 20.0 ng/ml.

4. Reference: Human standard preparation added to bovine serum.

5. RIA Procedure:
   - A. Begin and complete assay
     1. Pipette in non-coated polypropylene tubes
        - NSB – 100 µl of 0 Std
     2. Pipette in antibody coated tubes
        - 0 Std – 100 µl
        - Std – 100 µl
        - Ref – 100 µl
        - Unknowns – 100 µl
     3. Pipette 1 ml of ^{125}I –P4 provided in the kit to all tubes including two Total Count non-coated polypropylene tubes.
     4. Vortex tubes briefly and incubate at room temperature for 3 h.
     5. Pour off supernatant.
     6. Count radioactivity of each tube using a gamma counter.

VITA

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Education

B.S., Animal Science, University of Missouri - Columbia, Columbia, MO, 2003

M.S., Physiology of Reproduction, Texas A&M University, College Station, TX, 2006

Selected Publications

Cooper, D. A., J. A. Cartmill, J. P. Saldarriaga, J. F. Zuluaga, and G. L. Williams. 2005. Evidence that the attenuated release of LH accompanying transition into the non-breeding season in mares occurs without coincident suppression of pulsatile GnRH secretion. Nineteenth Equine Science Society Proceedings.

Saldarriaga, J. P., D. A. Cooper, J. A. Cartmill, R. L. Stanko and G. L. Williams. 2005. Performance of CIDR-based synchronization programs for timed AI in Brahman-influenced cattle. Proceedings Ninth Course on Advances in Production and Reproduction of Cattle, IRAC, Uberlandia, Brazil, pp. 233-242

Saldarriaga, J., D. Cooper, J. Cartmill, R. Stanko, G. Williams. Synchronization of ovulation for timed AI (TAI) in Bos indicus-influenced cattle using CIDR-based, GnRH-prostaglandin combinations I: ovarian follicular, luteal and hormonal events associated with suboptimal reproductive outcomes. Proceedings American Society of Animal Science, Annual Meeting, 2005

Saldarriaga, J., J. Zuluaga, J. Cartmill, D. Cooper, G. Williams. Synchronization of ovulation for timed AI (TAI) in Bos indicus-influenced cattle using CIDR-based, GnRH-prostaglandin combinations II: assessment of estrual and ovulatory distributions with Select Synch + CIDR to optimize TAI with Co-Synch + CIDR. Proceedings American Society of Animal Science, Annual Meeting, 2005